If I have the belief that I can do it, I will surely acquire the capacity to do it, even if I may not have it at the beginning.

- Mahatma Gandhi

Be of good cheer. Do not think of today's failures, but of the success that may come tomorrow. You have set yourself a difficult task, but you will succeed if you persevere; and you will find a joy in overcoming obstacles.

- Helen Keller

University of Alberta

Regulation of Vascular Function During Pregnancy: A Novel Role for the Pregnane X Receptor

by

Kathryn Anne Hagedorn ©

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Abstract

The mechanisms underling pregnancy-induced alterations in maternal vascular function are unclear. Progesterone metabolites, which are elevated during pregnancy, are potent ligands for the Pregnane X Receptor (PXR) a nuclear receptor which regulates cytochrome P450 (CYP) enzyme expression. CYP epoxygenases produce epoxyeicosatrienoic acids (EETs), known vasodilators. We hypothesized, therefore, that PXR contributes to the regulation of vascular tone during pregnancy by enhancing CYP mediated relaxation. PXR and *Cyp2c29* expression was shown in mouse mesenteric arteries by RT-PCR. Assessment of vascular function by wire myography showed blunted vasoconstriction and enhanced vasorelaxation in pregnant wildtype mice. However, these adaptations were absent in PXR knockout mice, indicating that PXR mediates the development of altered vascular function during pregnancy. Inhibition of CYP epoxygenases and EETs caused negligible reductions in vasoconstriction, however this pathway may still regulate vasorelaxation. Further investigation of these PXR mediated mechanisms will contribute significantly to our understanding of pregnancy-induced alterations in vascular function.

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Gloria Van Malsen

It was you who taught me the value of an education
and the dedication needed to pursue it.

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List of Abbreviations

ANOVA	analysis of variance
BK	bradykinin
BK_{Ca}	large conductance, calcium sensitive potassium channel
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
COX	cyclooxygenase
CYP	cytochrome P450 enzyme
DHETs	dihydroxyeicosatrienoic acids
5β-DHP	5beta-dihydroprogesterone
DMEM	Dulbecco's modified eagle's medium
DNA	deoxyribonucleic acid
DR	direct repeat
EDHF	endothelium-derived hyperpolarizing factor
EETs	epoxyeicosatrienoic acids
ER	everted repeat
IK_{Ca}	intermediate conductance, calcium sensitive potassium channel
IPR	IP receptor
IR	inverted repeat
MS-PPOH	N-methylsulphonyl-6-(2-propargyloxyphenyl) hexanamide
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
NO	nitric oxide
NOS	nitric oxide synthase
PE	phenylephrine
PKA	protein kinase A
PKG	protein kinase G
PXR	pregnane X receptor
RIN	RNA integrity number
RT-PCR	reverse-transcriptase polymerase chain reaction
SEM	standard error of the mean
SK_{Ca}	small conductance, calcium sensitive potassium channel
SUL	sulfaphenazole
VSM	vascular smooth muscle

CHAPTER I - INTRODUCTION

1. Introduction

During pregnancy, dramatic alterations in maternal physiology are necessary to support the developing fetus and ensure successful gestation. In particular, marked hemodynamic changes occur, including a 45-50% increase in plasma volume, with a concomitant increase in cardiac output of 30-50% over non-pregnant values[1]. Nevertheless, despite alterations which would otherwise elevate blood pressure, maternal arterial blood pressure decreases during pregnancy, reaching a nadir around 28 weeks' gestation and then gradually rising again as the pregnancy progresses[1-3]. Failure of the maternal cardiovascular system to adapt appropriately to the challenge of pregnancy occurs in up to 15% of pregnancies[4,5], and can result in disorders such as gestational hypertension (hypertension presenting after the 20th week of pregnancy) and preeclampsia (gestational hypertension with proteinuria)[182]. The etiology of these disorders, which can lead to both maternal and neonatal morbidity and mortality, is currently unknown. More significantly, the physiological mechanisms underlying the maternal cardiovascular adaptations to pregnancy in general also remain unclear. Although research into the mechanisms regulating maternal vascular tone during pregnancy has generated some insight, further knowledge in this area is required and may ultimately contribute to a greater understanding of the vascular pathologies which can arise during gestation.

Progesterone synthesis and metabolism is dramatically increased during gestation. Progesterone metabolites, in particular 5β-pregnane-3,20-dione (also known as 5β-dihydroprogesterone) are potent endogenous ligands for the Pregnane X Receptor (PXR)[6]. PXR is predominately expressed in the liver and intestines[6], although mRNA for this nuclear receptor has also been found in other tissues including rat brain capillaries[7]. Functionally, PXR is primarily involved in the metabolism of both xenobiotics as well as endogenous compounds via the induction of cytochrome P450 (CYP) enzymes[8]. PXR can also induce the expression of other genes involved in drug metabolism, such as conjugating and transporter enzymes[9], and recently has been shown to regulate the expression of inducible nitric oxide synthase (iNOS)[10]. Interestingly, in addition to drug metabolism, CYPs have also been suggested to play a role in the mediation of vascular tone, as metabolism of arachidonic acid by CYP enzymes can produce both vasodilator and vasoconstrictor eicosanoids. We therefore have developed a series of studies designed to investigate the possibility that PXR may participate in the regulation of vascular tone during pregnancy via the induction of vasodilator producing CYP enzymes (figure 1).

2. Cardiovascular adaptations to pregnancy

The body undergoes countless physiological changes in response to pregnancy. The means by which the cardiovascular system adapts to the demands of pregnancy are briefly outlined below, while specific vascular adaptations are discussed in section 3.

The paradoxical observation of decreased arterial pressure in the face of increased plasma volume and cardiac output can be attributed to reductions in systemic vascular tone, which occur early in gestation and parallel the changes seen in maternal blood pressure[3, 11-13]. Decreasing as much as 21% from early to mid-pregnancy and then returning to near normal values at term[1], reductions in systemic vascular tone increase vascular capacitance and reduce afterload, triggering increases in maternal heart rate and cardiac output to compensate. In the kidneys, decreased vascular tone results in arteriolar dilation, causing an increase in renal blood flow and glomerular filtration rate. Additionally, sodium reabsorption rates also increase, resulting in overall fluid retention and plasma volume expansion[12,13]. In the non-pregnant state, these compensatory mechanisms would serve to reinstate pressure homeostasis. However, in pregnancy, vascular responsiveness is shifted towards enhanced vasodilation, thereby preventing maternal blood pressure from rising. This pregnancy-induced shift in vascular function during pregnancy comprises both an increased response to vasodilators and a diminished response to vasoconstrictors. While not yet completely understood, it is believed that these changes are due, in part, to alterations in the endothelial regulation of vascular tone.

3. Endothelial regulation of vascular relaxation

Vascular tone is determined by the degree of constriction of the vascular smooth muscle (VSM). Constriction of the VSM is regulated by a complex array of vasoconstrictor and vasodilator agents, including neural, local and plasma borne molecules which themselves can be regulated by physical stimuli such as shear stress, pressure and hypoxia[14,15]. The endothelium is a single layer of cells that line the lumen the vasculature, acting as an interface between the circulating blood and the vascular tissue. At any given time, a vessel receives a variety of constricting and/or relaxing signals. The endothelium integrates these signals and regulates VSM constriction by creating a paracrine milieu of both constricting and relaxing factors, the balance of which determines vascular tone. The signaling pathways by which the endothelium regulates vascular tone are multiple and complex, therefore this discussion which follows is limited to only those most pertinent to this project, the endothelium derived relaxing factors.

The endothelium-derived relaxing factors include prostacyclin, (PGI_2) nitric oxide (NO), and an as yet unidentified endothelium-derived hyperpolarizing factor or factors (EDHF). Each plays a role in maintaining tone. However, the individual contribution of any one factor to vasorelaxation depends on size of vessel, with NO playing a proportionately greater role in larger conduit vessels and regulation by EDHF becoming more predominant as vessel diameter decreases[16]. During pregnancy, the shift in vascular response towards enhanced vasodilation and decreased constriction is thought to be due, in part, to an increase in the release of relaxing factors from the endothelium.

3.1. Prostaglandins

Prostaglandins, in particular prostacyclin (PGI_2), were the first endothelium-derived relaxing factor to be discovered, when, in 1976, Moncada et al who reported on their anticlotting and vasorelaxant properties[17]. Prostaglandins are a family of eicosanoids produced in a two step process that begins with the conversion of arachadonic acid into an unstable, cyclic intermediary, PGG_2, by the cyclooxygenase (COX) enzyme (also known as PGHS). Three COX enzymes exist, COX-1, which is constitutively expressed, COX-2 which is inducible, and COX-3, which is a splice variant of COX-1 and is also constitutively expressed. The second step in the pathway involves the conversion of PGG_2 by specific isomerases into the vasodilators PGI_2, PGD_2 and PGE_2, and the vasoconstrictors, thromboxane A_2, and PGF_2. Of these prostanoids, PGI_2 is the major isoform produced by endothelial cells[18]. PGI_2 achieves vasorelaxation following receptor mediated endothelial cell stimulation by diffusing to the underlying smooth muscle where it activates G proteins, causing an increase in the expression of cyclic AMP (cAMP). Increased cAMP in turn activates protein kinase A (PKA), ultimately decreasing the VSM intracellular calcium concentration and causing vasorelaxation (figure 2). Although not its primary mechanism of action, PGI_2 can also cause vasodilation via cAMP mediated opening of large conductance potassium channels (BKca) [19] and ATP sensitive potassium channels (K_{ATP})[20] which hyperpolarizes the VSM. Hyperpolarization in turn reduces the open probability of voltage dependent calcium channels and decreases the turnover of intracellular phosphatidylinositides, which together decrease intracellular calcium levels, bringing about vasorelaxation[21]. Overall, relative to vascular function, PGI_2 appears be involved in the maintenance of blood pressure by enhancing vasodilation, especially during disease states such as hypertension and atherosclerosis. Additionally, because of its antithrombotic properties, PGI_2 also plays an important role during the inflammatory process by mediating platelet adhesion to the endothelium[18].

Relative to vascular function during pregnancy, although there is an increase in 6-keto-$PGF_{1\alpha}$ (a stable metabolite of PGI_2) in gestation, PGI_2 does not seem to be a major factor in generating enhanced vasodilation during pregnancy[3]. This was first suggested by Conrad and Colpoys in 1986 who found that vasodilatory prostaglandins did not mediate the attenuation of the systemic and renal pressor responsiveness observed during pregnancy[22]. Similarly, Luksha et al. recently investigated the mechanism of endothelial dependent vasodilation in subcutaneous small arteries from healthy pregnant women and found that PGI_2 was not involved in mediating endothelium dependent dilation induced by stimulation with bradykinin[23].

There is some suggestion however, that impaired PGI_2 activity may partially underlie the altered endothelial function of preeclampsia, in that there is a reduction in 6-keto-$PGF_{1\alpha}$ plasma levels in preeclamptic women compared to normal pregnant women[24].

3.2. Nitric Oxide

In 1980, Furchgott and Zawadzki identified a labile, diffusible molecule that was produced by the endothelium and caused vascular smooth muscle relaxation[25]. This molecule, which they called endothelium-derived relaxing factor, was subsequently proven to be the free radical NO and has come to be known as one of the principal mechanisms by which the endothelium regulates the VSM. Nitric oxide is an L-arginine derivative, produced by one of three nitric oxide synthase (NOS) enzymes: a neuronal form, nNOS (NOS1), an inducible form, iNOS (NOS2), and a constitutive form, eNOS (NOS3), which is the most common NOS isoform expressed in the endothelium. NO also elicits vasorelaxation by diffusing into the underlying VSM where it activates soluble guanylate cyclase to increase cyclic GMP (cGMP) expression. Upregulation of cGMP results in the activation of a cGMP dependent kinase and subsequently a decrease in intracellular calcium, which causes diminished actin-myosin binding and ultimately vasorelaxation (figure 2). Increased cGMP also causes a reduction in the sensitivity of the contractile apparatus to calcium by activating myosin light chain phosphatase[26]. Furthermore, NO can also cause vasorelaxation via VSM hyperpolarization following cGMP dependent activation of potassium channels. Specifically, NO has been shown to activate BK_{Ca} channels in the placenta[27] and human cutaneous arteries[28], K_{ATP} channels in rabbit mesenteric arteries[183] and Kv channels in pulmonary artery smooth muscle cells[184]. NO can also produce hyperpolarization via cGMP independent mechanisms by acting on K^+ channels directly[29, 30].

NO is known to play a major role in the vascular adaptations to pregnancy[31] although its involvement is vascular bed specific. Endogenous production of NO has been shown to be increased in gravid rats[32, 33]. Furthermore, evidence from studies using uterine and mesenteric arteries from both rats and rabbits show that the contribution of NO to vasodilation under both basal and stimulated conditions is increased during pregnancy[34-36]. This is in agreement with results obtained from resistance myometrial arteries from term pregnant women which showed increased NO mediation of vasodilation induced by shear stress and blunted norephinephrine induced constriction[37]. Furthermore, increased expression of all NOS enzymes in the renal vasculature is believed to be involved in mediating the renal hemodynamic changes that occur during pregnancy[33, 38].

3.3. Endothelium derived hyperpolarizing factor

As efforts to understand the mechanism of endothelium-dependent vasorelaxation progressed through the 1980s, it was discovered that a third pathway, independent of NO and PGI_2, existed by which the endothelium can induce vasorelaxation[39]. This endothelium-dependent vasorelaxation resulted from VSM hyperpolarization and was therefore said to be due to an endothelium-derived hyperpolarizing factor (figure 2). As research into the nature of EDHF progressed however, this terminology has actually been quite misleading, since it has become increasingly apparent that EDHF is in fact not one single substance but rather that multiple agents are capable of eliciting (or working together to elicit) NO and PGI_2 independent vasorelaxation. These studies were further confused by the fact that EDHF responses can differ relative to the species, type of vessel and vascular bed studied, as well as to the agonist used for endothelial stimulation[40]. Despite these challenges, continued efforts to unravel the mysteries of EDHF have resolved the phenomenon of endothelium-derived VSM hyperpolarization into two distinct categories: one which requires the activation of small (SK_{Ca}) and intermediate (IK_{Ca}) conductance calcium dependent K^+ channels and endothelial hyperpolarization, and another which results from the release of factors from the endothelium capable of causing VSM hyperpolarization independent of endothelial hyperpolarization[41]. To avoid the confusion which has plagued the field to date, Feletou and Vanhoutte propose to call the former simply endothelial-derived hyperpolarizations or EDHF-mediated responses and to call the latter by their proper name as they are each confirmed to induce vasorelaxation via VSM hyperpolarization[41, 42].

3.3.1. Endothelium-derived hyperpolarization

Historically, the EDHF response has been characterized as being sensitive to inhibition by the combination of apamin, an inhibitor of small conductance potassium channels ($\mathrm{SK_{Ca}}$), and charybdotoxin, a non-specific inhibitor of intermediate ($\mathrm{IK_{Ca}}$) and large conductance ($\mathrm{BK_{Ca}}$) potassium channels. Iberiotoxin, a specific inhibitor of $\mathrm{BK_{Ca}}$, cannot substitute for charybdotoxin, implicating $\mathrm{SK_{Ca}}$ and $\mathrm{IK_{Ca}}$ channels in the EDHF response[39, 43]. Paradoxically, $\mathrm{SK_{Ca}}$ and $\mathrm{IK_{Ca}}$ are predominantly expressed on endothelial cells whereas $\mathrm{BK_{Ca}}$ channels are mostly expressed on the VSM[42, 44]. The fact that the K^+ channels identified as being involved in the EDHF response were located on the endothelium as opposed to on the VSM at first appeared to be incompatible with knowledge that EDHF mediated vasorelaxation occurs via hyperpolarization of the VSM. However, it is now known that endothelial hyperpolarization represents a critical first step in EDHF-mediated responses. Stimulation of endothelial G-protein coupled receptors causes a transient increase in the intracellular calcium concentration of the endothelium, leading to the activation of $\mathrm{SK_{Ca}}$ and $\mathrm{IK_{Ca}}$ channels and subsequent endothelial hyperpolarization[21, 42].

The induction of VSM hyperpolarization by endothelial hyperpolarization is thought to occur through two distinct pathways, the direct transmission of hyperpolarization via gap junctions, and the accumulation of K^+ in the space between the endothelium and the VSM. Endothelial cells are electrically coupled to VSM cells via myo-endothelial gap junctions (MEGJ). Gap junctions are aqueous pores formed by the assembly of six connexin subunits within the plasma membrane. Docking of connexin complexes on adjacent cells provides a passageway for the transfer of ions and polar molecules from the cytoplasm of one cell to another[45]. Gap junctions have been implicated in the mediation of endothelium-derived hyperpolarization in a variety of tissues, some of which include rat middle cerebral[46], hepatic[47], and mesenteric[47, 48] arteries, guinea pig internal carotid arteries[47] rabbit iliac arteries[49], porcine coronary arteries[50] and human subcutaneous[23] and myometrial[51] arteries. Inhibition of the myoendothelial gap junctions in these vessels attenuated or abolished VSM hyperpolarization but did not prevent endothelial hyperpolarization upon stimulation, indicating that the electrical coupling of the endothelium to the VSM is responsible for at least a portion of the EDHF response. Interestingly, the number of myoendothelial gap junctions increases as vessel diameter decreases[42], suggesting that the size of the myoendothelial gap junctions population provides a basis for the observation that the EDHF component of vasorelaxation is greater in smaller caliber vessels than in larger ones[48].

In addition to the direct electrical coupling of the endothelium to the VSM, the EDHF-mediated response can also be attributed to the K^+ ion itself, which accumulates in the myoendothelial space following endothelial stimulation[42, 52]. K^+ released through endothelial SK_{Ca} and IK_{Ca} channels upon stimulation elevates the myoendothelial K^+ concentration from 4.6 mM to 11.6 mM[52]. Normally, elevations in extracellular K^+ would degrade the K^+ electrochemical gradient, promoting VSM depolarization and vasoconstriction. The VSM however, expresses inwardly rectifying K^+ channels (K_{IR}), which, when exposed to increases in extracellular K^+ between 8 - 15 mM, cause membrane hyperpolarization and vasodilation[53]. Interestingly, the conductance through K_{IR} channels increases as arterial diameter decreases, again providing a basis for the increased proportion of EDHF regulation of vascular tone in small caliber vessels[53]. Additionally, the VSM electrogenic Na^+-K^+ pump can also be activated by elevations in extracellular K^+[54] and has been implicated along with K_{IR} as the mechanism by which endothelial K^+ efflux induces VSM hyperpolarzation[52, 55].

In summary, as is depicted in figure 3, vasorelaxation due to endothelial-derived hyperpolarization arises following SK_{Ca} and IK_{Ca} channel dependent hyperpolarization of the endothelium. Hyperpolarization is then induced in the VSM either by direct transmission via myoendothelial gap junctions or by the paracrine activation of VSM K_{IR} channels and Na^+-K^+ pumps via an accumulation of extracellular K^+ from the endothelium.

3.3.2. Other endothelium-derived hyperpolarizing substances

Further to the EDHF-mediated mechanism of VSM hyperpolarization discussed above, many factors released by the stimulated endothelium can elicit VSM hyperpolarization. The release of these factors is not dependent upon hyperpolarization of the endothelium, allowing their activity to be differentiated from the effects of K^+ ions alone. As was indicated previously, both PGI_2 and NO can activate K^+ channels on the VSM, although this is generally not considered to be their primary mechanism of action. Recently, however, a number of other substances have been identified as possible endothelial derived hyperpolarizing substances. These include CYP epoxygenase metabolites of arachidonic acid, the epoxyeicosatrienoic acids (EETs), as well as hydrogen peroxide and C-type natriuetic peptide[42]. Like all EDHF research, efforts to definitively identify each as an endothelium-derived hyperpolarizing substance remains controversial and there is data to both support and refute the claim for each. However, despite the sometimes conflicting results, strong evidence has emerged to support the conclusion that EETs produced by CYP enzymes can be involved in endothelium-derived hyperpolarization dependent vasorelaxation. Indeed, some of the earliest EDHF studies suggested that CYP metabolites of arachidonic acid could induce VSM hyperpolarization dependent vasodilation[39], while more recently, advanced technologies have helped to elucidate the identity of individual CYPs and EETs involved. As one of the major areas of focus for this project, the role of CYP epoxygenases and EETs in the mediation of vascular tone is detailed in section 4, including a discussion of both the evidence to support and refute the argument that CYP metabolites of arachidonic acid are endothelial-derived hyperpolarizing substances.

3.3.3. *Role of endothelium-derived hyperpolarization and endothelium-derived hyperpolarizing substances in the regulation of vascular tone*

Regardless of the nature of EDHF (used here to collectively refer to vasorelaxation due to both endothelium-derived hyperpolarization and endothelium-derived hyperpolarizing substances), functionally, it is known to play an important role in the regulation of vascular function. EDHF has been shown to be involved in the regulation of vascular tone in both *in vitro* and *in vivo* preparations from a variety of vessels (coronary, cerebral, and renal arteries as well as subcutaneous, penile, cremaster, mesenteric, and hind limb arterioles), and in a number of different species (humans, rats, pigs, dogs, cows and guinea pigs) [reviewed in [56]]. Physiologically, it is postulated that EDHF plays a role in the control of blood flow[41], particularly in situations such as the maintenance of arterial blood pressure, penile erection, exercise hyperemia and hypoxia[41]. Nevertheless, despite the demonstrated involvement of EDHF in the elicitation of vasorelaxation, a certain degree of controversy exists about whether EDHF works in conjunction with NO to mediate vasodilation or if it represents instead a redundant, back-up system to be called into play in the event endothelial dysfunction[41]. Certainly, given the multiplicity of factors involved in EDHF, both are possible. There is, however, evidence to suggest that NO inhibits EDHF[57]. Interestingly, gender differences have been reported for the regulation of vascular relaxation by EDHF[58-60], with females demonstrating a more significant EDHF response than males. In particular, NOS inhibition attenuated vasorelaxation to a lesser degree in females than in males, a characteristic that was attributed to the females' greater capacity for EDHF mediated relaxation. The authors propose that these findings support a role for EDHF in the maintenance of vascular function in the absence of NO and suggest that such a mechanism may represent a explanation for the protective effects of gender against cardiovascular disease[59].

Relative to vascular function during pregnancy, EDHF has also been shown to be enhanced by gestation in both humans and animals[3, 61-64]. Furthermore, Kenny et al. have suggested that the endothelial dysfunction associated with pre-eclampsia is due, in part, to the loss of EDHF function[3, 62]. Isolated vessel studies have been done to investigate type of EDHF involved. Gap junctions[23, 51], K_{IR} channels[65] and CYP epoxygenase metabolites[66, 67] have all been implicated in the mediation of this enhanced VSM hyperpolarization dependent relaxation. As was indicated previously, NO mediated regulation of vascular tone is also enhanced in response to pregnancy. Given that NO can attenuate EDHF release, these two observations appear to be in conflict. However, when considered in light of the fact that NO and EDHF act predominantly in large and small caliber vessels respectively, it is evident that together NO and EDHF form a powerful mechanism by which pregnancy adaptations are induced throughout the entire vascular system.

4. CYP epoxygenases and the regulation of vascular tone

CYP enzymes are heme containing, NADPH dependent monoxygenases that are classified into a superfamily of proteins. This superfamily is further categorized into families whose members have at least 40% homology in their amino acid sequence (indicated by an arabic numeral), and subfamilies whose members share >55% homology (indicated with a letter)[68]. The nomenclature for CYP enzymes is also quite complex, with CYP proteins from all species except mice being indicated by capital letters (eg: CYP2C9), while mouse CYP proteins are denoted with only a capital C (eg: Cyp2c37). The same convention applies when discussing CYP genes, except that the names are italicized.

In eukaryotes, CYPs are membrane bound proteins, anchored to the endoplasmic reticulum and inner mitochondrial membranes by their N-terminal. Although they are primarily found in the liver, CYPs are also expressed extrahepatically, in a variety of other tissues including blood vessels[69]. Functionally, CYPs are responsible for the metabolism of xenobiotics as well endogenous substances such as cholesterol, steroids, bile acids, vitamin D and other lipids including arachidonic acid. Indeed, CYPs represent a third pathway of eicosanoid synthesis, in addition to the activity of cyclooxygenase and lipoxygenase enzymes (figure 4). Specifically, metabolism of arachidonic acid by CYP epoxygenases produces EETs. EETs are predominantly vasodilatory in nature, although they can also be vasoconstrictors, and as such are generally considered to be an endothelium-derived hyperpolarizing substance. Metabolism of arachidonic acid by another type of CYP, the ω-hydroxylases, produces vasoactive eicosanoids of another type. Hydroxyeicosatetraenoic acids, formed by the terminal hydroxylation of arachidonic acid, are chiefly vasoconstrictors[70] that are postulated to also be involved in the regulation of vascular tone by working in opposition to EETs and other endothelium-derived relaxing factors.

Studies investigating the role of CYP epoxygenases and the EETs in the regulation of vascular tone have been ongoing since the mid-1980s. As is discussed below, evidence exists both for and against the idea that they are involved in endothelium-derived hyperpolarization, again supporting the concept that EDHF and its underlying mechanisms differ across vascular beds, species and physiological contexts.

4.1. CYPs can produce endothelium-derived hyperpolarizing substances

Many methods have been used to investigate the involvement of CYP enzymes in endothelial dependent relaxation. Early work showed that both acute and chronic CYP inhibition attenuated vasorelaxation in rabbit pulmonary[71] and canine coronary arteries[72] whereas induction of CYP expression enhanced vasodilation in these arteries. Similarly, CYP inhibition decreased acetylcholine induced vasodilation in rat mesenteric arteries[73] and the canine coronary microcirculation[74]. Additionally, acute CYP inhibition increased coronary perfusion pressure in isolated rat hearts[75, 76] and enhanced renal afferent arteriole constriction[77]. In humans, CYP inhibition *in vivo* attenuated nicorandil induced[78] and bradykinin induced[79] increases in forearm blood flow. *In vitro*, CYP inhibition attenuated both coronary arteriolar dilation and hyperpolarization of the VSM[80] and decreased acetylcholine induced vasorelaxation in subcutaneous resistance arteries[81].

The ability of CYP enzymes to mediate VSM hyperpolarization has also been confirmed. Working with native and cultured porcine coronary endothelial cells, Popp et al. showed that, in the presence of both NOS and COX inhibition, cells stimulated with bradykinin released an agent which hyperpolarized VSM[82]. This same agent was also able to activate calcium dependent K^+ channels. CYP inhibition with either clotrimazole or 17-octadecynoic acid abolished these effects, although these results should be interpreted with caution since clotrimazole can also inhibit K^+ channels directly. Finally, treatment of these cells with β-naphthoflavone induced CYP expression, and increased release of the hyperpolarizing factor[82].

A number of CYP enzymes have epoxygenase activity, primarily those of the CYP2 gene family, although CYP1A and 4A can also produce EETs[69, 83]. Many of these are expressed in vascular tissue, with the 2C, 2E and 2J as well as the 1A isoforms having been identified in bovine coronary arteries[84]. CYP2C11, 2J3, 2J4, and 4A3 isoforms have been detected in rat mesenteric arteries with rat renal arterioles additionally expressing CYP2C23, 4A1 and 4A2 but not 2C11[85, 86]. Moreover, work with rat and porcine endothelial cells and rat arterioles further identified the CYP1A, 2C and 2J isoforms as being present in the endothelium[87, 88]. In 1999, the CYP2C8/9 isoforms were termed an EDHF synthase based on work that showed that induction of these isoforms (murine Cyp2c29 and Cyp2c37 respectively) with β-naphthoflavone both increased 11,12-EET production and caused EDHF mediated hyperpolarization and relaxation in porcine coronary arteries[87]. This vasorelaxation was attenuated when arteries were incubated with sulfaphenazole, an inhibitor which acts primarily on the CYP2C9 isoform, although it is also weakly antagonistic for CYP2C8 and CYP2C19. Additionally, inhibition of CYP2C8/9 using antisense oligonucleotides prevented VSM hyperpolarization. Subsequently, this group also reported that antisense oligonucleotide inhibition of CYP2C8/9 also attenuated EDHF mediated vascular responses in resistance arteries from the hamster gracilis muscle[89].

4.2. EETs can be endothelium-derived hyperpolarizing substances

There are four EET regioisomers: 5,6-EET, 8,9-EET, 11,12-EET and 14,15-EET (figure 5). Research suggesting that EETs produced by CYP epoxygenases were responsible for a portion of non-NO/non-PGI vasorelaxation has been accumulating since the mid 1990s, when exogenous application of EET isomers was found to relax bovine[90] and porcine[91] coronary arteries. In 1996, work by Campbell et al. identified EETs as EDHFs when they showed that CYP inhibition with SFK 525A and miconazole attenuated methacholine induced vasorelaxation of bovine coronary arteries, and that methacholine induced the production of EETs from the endothelium of perfused coronary arteries[92]. They also showed that all EET isomers cause both vasorelaxation and VSM hyperpolarization, and that EETs increase the open probability of K^+ channels. Other researchers have since reported similar findings, observing that stimulation with bradykinin not only resulted in vasodilation, but also promoted EET production in renal afferent arterioles[93]. Furthermore, both EETs and their metabolites the dihydroxyeicosatrienoic acids (DHETs) (produced when soluble epoxide hydroxylase converts EETs to their corresponding diols) have also been reported to be potent vasodilators in the canine coronary microcirculation[94]. Similarly, in bovine coronary arteries, 14,15-DHET also induces vasorelaxation, although it is less potent that 14,15-EET[95].

4.2.1. Mechanism by which EETs induce VSM hyperpolarization

The mechanism by which EETs act as endothelium-derived hyperpolarizing substances remains to be fully determined, as it is likely that EETs can stimulate VSM hyperpolarization via a number of different pathways (figure 6). One possibility is that EETs, produced by CYPs in the endothelium upon stimulation act as extracellular messengers by diffusing across the myoendothelial space and inducing VSM hyperpolarization. In support of this, 14,15-EET has recently been shown to represent a transferable EDHF since 14,15-EET produced by an endothelium-intact donor artery can elicit vasorelaxation in an adjacent endothelium-denuded detector artery[96]. Induction of VSM hyperpolarization by EETs in this manner is purported to occur via the opening of K+ channels in the VSM[97]. The type of K^+ channel activated by EETs is largely reported to be the BK_{Ca} channel[98-104], although 11,12-EET has also been shown to activate ATP-sensitive K+ channels[105]. How EETs act as extracellular messengers is also currently being investigated. There is evidence to suggest that EETs act through a receptor on the VSM plasma membrane. In particular, EETs exposed to the extracellular but not the cytosolic surfice of cells increased the open probability of K+ channels, which the authors suggest may indicate a receptor-mediated interaction [97]. While not yet identified, research by Snyder et al. suggests the presence of a receptor for 14,15-EET on the surface of VSM cells[106]. These researchers attached a synthetic 14,15-EET derivative to silica beads, which prevented it from entering VSM cells in culture but did not alter its biological activity, suggesting that its effects might be initiated via an extracellular receptor. Wong et al. have also reported high affinity binding sites for 14,15-EET on guinea pig monocytes[107]. Moreover, Li and Campbell have shown that effects of 11,12-EET on the BK_{Ca} channel are mediated via a G-protein, supporting the possibility that EET induced activation of the BK_{Ca} channel occurs via an intracellular signaling pathway[108].

In addition to acting on the VSM directly, EETs are also proposed to act as intracellular messengers by potentiating changes within the endothelium itself. This can occur in a number of ways. For example, EETs have been shown to enhance endothelial intracellular Ca^{++} concentrations by activating transient receptor potential cation channels in the endothelium[109]. They have also been shown to modulate the activity of intraendothelial gap junctions[110]. Clearly the involvement of EETs in the regulation of vascular tone is a complex phenomenon which is not yet fully understood. Nevertheless, taken together, these studies support the conclusion that EETs can act as endothelium-derived hyperpolarizing substances.

4.3. CYPs/EETs do not always mediate EDHF dependent relaxation

While there is compelling evidence to indicate that CYP enzymes and their metabolites do represent an EDHF or a component of the EDHF signaling pathway, evidence to the contrary also exists. For example, inhibition of CYP activity did not alter acetycholine induced VSM hyperpolarization in the rat superior mesenteric artery[111] nor did it alter vasodilation in small porcine retinal arterioles stimulated with bradykinin[112]. The researchers who reported the EDHF synthase activity of CYP2C have also reported that inhibition of multiple CYP enzymes does not alter the vascular responses of wildtype or eNOS knockout mice[113]. Similar results using the same mouse model were also seen by Chlopicki et al.[114]. Finally, CYP inhibition by sulfaphenazole did not alter vasorelaxation in either human mesenteric or renal arteries[115, 116]. Differences in experimental set up, specificity of the inhibitor, and the type of tissue tested may provide possible explanations for these discrepant observations. Alternately, in keeping with the differential nature of EDHF, they may truly reflect an absence of CYP involvement in the EDHF component of vascular function in these situations.

4.3.1. Production of Reactive Oxygen Species by CYP enzymes

Recently, it has been reported that inhibition of CYP2C9 improves NO mediated vasorelaxation in people with coronary artery disease[117]. The authors suggest that this improvement may be the result of reduced production of reactive oxygen species. Because CYP2C9 is a monoxygenases, it produces reactive oxygen species as a byproduct during reactions such as epoxygenation[118]. Indeed, CYP2C9 itself has been shown to be a functionally significant source of reactive oxygen species in coronary arteries[119]. Relative to endothelial function, reactive oxygen species present a hazard to the endothelial cell in that they can scavenge NO, thereby reducing vasodilation. This apparent dichotomy between the fact that enzymes which produce vasodilators also produce substances which hinder vasodilation can be resolved by the fact that the body has developed extensive mechanisms to eliminate reactive oxygen species[120]. In situations of endothelial dysfunction, however, such as occurs in patients with coronary artery disease, the capacity of the cell to deal with reactive oxygen species may be diminished or there may be such an increase in reactive oxygen species production that it overwhelms the cellular antioxidant machinery. Thus, it is possible that although the activity of this enzyme may contribute to the production of vasodilators, the concomitant production of reactive oxygen species may in fact represent a detrimental component of CYP2C9 activity relative to vascular function.

Interestingly, CYP2J2, which is expressed predominantly in cardiac and vasculature tissue and which is capable of producing EETs, has recently been shown to be not a functionally significant source of reactive oxygen species[186, 187]. Furthermore, polymorphisms of this gene, which result in decreased in protein expression and diminished EET production, are considered a risk factor for the development of coronary artery disease[125]. Taken together, the differential ability of CYP epoxygenase isoforms to produce reactive oxygen species speaks to the delicate balance which exits between appropriate vascular function and oxidative responses and suggests an area for further clinical investigation.

4.4. Functional significance of CYPs and EETs

4.4.1. CYP/EET involvement in cardiovascular disease

Although many details regarding the mechanisms by which CYP epoxygenases and their metabolites regulate vascular tone are as yet unresolved, they have already been shown to play significant roles in the mediation of cardiovascular function. For example, the attenuated vasoconstriction which results following chronic hypoxia has been attributed to enhanced CYP2C9 expression and activity[121]. Also, treatment of spontaneously hypertensive rats with γ-linolenic acid (a polyunsaturated fat which can be converted to arachidonic acid) lowers systolic blood pressure[122], elevates renal CYP2C and 2J protein expression and increases renal epoxide EET production[123]. Conversely, situations of impaired cardiovascular function are associated with impaired CYP epoxygenase function. In obese Zucker rats, endothelial dysfunction is associated with decreased epoxygenase expression in both mesentereic and renal arteries[85] while in insulin resistant rats, pentobarbital induction of CYP enzymes partially resolved endothelial dysfunction and normalized arterial pressure[124]. Finally, as was indicated previously, polymorphisms of the CYP2J2 gene in humans are associated with decreased plasma levels of EET metabolites and an increased risk of coronary artery disease[125].

4.4.2. CYP/EET modulation of vascular tone during pregnancy

There are a number of studies to suggest that CYPs and EETs may play a role in the regulation of vascular tone during pregnancy. Work done in aortas[66] and mesenteric arteries[67] from gravid rats showed that non-NO/non-prostanoid relaxation can be abolished with the CYP inhibitors, Clotrimazole and proadifen respectively, suggesting that this portion of relaxation is attributable to a CYP metabolite. Similar work done in uterine arteries from pregnant rats[65] and subcutaneous arteries from pregnant women[23], however, showed no change in the portion of relaxation due to EDHF during treatment with Miconazole and Sulfaphenazole respectively, possibly indicating that CYPs are not involved. Relative to the renal adaptations to pregnancy, EETs and their metabolites, the dihydroxyeicosatrienoic acids (DHETs) have been shown to be increased in the urine of pregnant women compared to non-pregnant women[126]. Additionally, Zhou et al. have recently shown that renal microsomes from pregnant rats exhibit increases in both CYP expression and the production of EETs as gestation progresses[127]. Moreover, they also showed that when treated with an IV infusion of the CYP inhibitor, 6-(2-propargyloxyphenyl)hexanoic acid (PPOH), pregnant rats experience a significant increase in maternal mean arterial pressure and a significant decrease in fetal pup weight. Taken together, these studies suggest that CYP enzymes and their metabolites may play a role in the mediation of vascular responses during pregnancy, and indicate a need for further investigation in this area.

5. The Pregnane X Receptor

The Pregnane X Receptor (PXR; NR1I2) is a nuclear receptor that can regulate the expression of some CYP enzymes at the genomic level (see section 5.4). Also known as the steroid and xenobiotic receptor[128] or the pregnane-activated receptor[129], PXR was first cloned in 1998 by Kliewer et al.[6], who identified two isoforms of this protein, PXR.1 and PXR.2. Original studies found PXR to be abundantly expressed in the liver and intestines[6, 128-131] as well as weakly expressed in the stomach and kidney[6]. Although, recently PXR mRNA has also been detected in mouse uterus, ovary and placenta[131], human breast[132] and lung[130], and rat brain capillaries[7]. This pattern of predominantly hepatic expression is reflective of PXR's role as what some have called the master regulator of homeland defense[133]. By monitoring the circulation for xenobiotics and endogenous substances and inducing the expression of CYPs, conjugating enzymes and transporters in response[7, 134-136], PXR promotes the elimination of potentially harmful substances and plays a major role the mediation of systemic detoxification. Increasingly, however, it is becoming apparent that PXR function is not limited solely to detoxification, but rather that it also plays important roles in maintaining overall homeostasis.

5.1. PXR is a ligand activated transcription factor

As a nuclear receptor, PXR is a ligand activated transcription factor. However, unlike most nuclear receptors, PXR is capable of being activated by a wide variety of substances, including xenobiotics such as phenobarbital, rifampicin, dexamethasone, nifedipine, taxol and hyperforin (St. John's wort)[137] as well as endogenous lipophilic compounds including progesterone metabolites[6, 129], glucocorticoids [6, 129], sterols[138] and bile acids[8]. Of the latter, the most potent are the pregnanes, in particular 5β-pregnane-3,20-dione[6]. Furthermore, in addition to interacting with a variety of structurally diverse molecules, PXR is also unusual amongst the nuclear receptors in that the specificity of its ligand binding profile differs significantly among species. For example, the murine but not the human isoform of PXR is strongly activated by the antiglucocorticoid, pregnenolone-16α-carbonitrile (PCN), and rifampicin can activate rabbit but not rat PXR[139]. The elucidation of the PXR crystal structure provided some explanation for these unusual characteristics.

5.2. Structural characteristics of the PXR protein

The structure of PXR is comprised of four characteristic regions common to all nuclear receptors: the modulator domain, the DNA binding domain, the hinge region and the ligand binding domain[140] (figure 7). An examination of the PXR ligand binding domain reveals a number of unique features to which the promiscuity and species specificity of PXR can be attributed.

5.2.1. Promiscuity of PXR ligand binding

In contrast to other nuclear receptors, the PXR ligand binding pocket is not only unusually large but also flexible, allowing it to range in size from 1280 to >1600 $Å^3$ [134]. This appears to be due to the presence of a novel 60 amino acid sequence located adjacent to the ligand binding pocket of PXR. This insert alters the oligomerization state of the receptor, allowing it to not only bind small ligands but also to expand to accommodate much larger molecules[134, 141]. Additionally, the bottom of the PXR ligand binding pocket also contains three regions which are mobile and can become disordered in order to facilitate contact with distinctly shaped ligands[137]. Finally, the ligand binding pocket of PXR has also been shown to "breathe", allowing small molecules to sample multiple modes of interaction by binding with PXR in up to three different orientations[137, 141]. Together, these unique modifications enable PXR to interact with a much wider range of ligands than most nuclear receptors and facilitate its ability to mediate the elimination of a diverse array of structurally dissimilar compounds.

5.2.2. Species differences in the PXR ligand binding profile

The reason for the differences in binding affinity between species can also be found in the ligand binding domain. A comparison of human, rat, mouse and rabbit PXR amino acid sequences shows a high degree of divergence, with only ~80% similarity between the human sequence and any of the others[139]. The key difference seems to involve 5 residues which line the interior of the ligand binding pocket[8]. Point mutation of these residues in murine PXR to those found in the human sequence resulted in a human activation profile for the mutated protein[141]. These differences are thought to be the result of divergent evolution[139], enabling each species to specialize and induce the expression of metabolizing enzymes in response frequently encountered toxins.

5.3. Ligand binding and PXR activation

Ligand binding activates PXR. Again, there appear to be species differences regarding where this occurs. In the mouse, when not ligand bound, PXR is localized to the cytoplasm, only moving into the nucleus upon activation[142]. In the human though, it would appear that PXR permanently resides in the nucleus[143, 144]. Nevertheless, regardless of where PXR is when ligand binding occurs, once activated it forms a heterodimer with the retinoid X receptor and concomitantly binds to the promoter region of its target gene via its DNA binding domain. The DNA binding domain is highly conserved amongst nuclear receptors[145]. Composed of approximately 70 amino acids that fold to form two DNA binding zinc fingers, DNA binding domains recognize specific nucleic acid sequences known as response elements. Response elements consist of a pair of hexameric sequences that exist as either direct repeats (DR), everted repeats (ER) or inverted repeats (IR) separated by one to nine nucleotides[146] as indicated by the numeral following the hyphen in the name of the response element (eg. ER-6 is separated by six nucleotides). Together with RXR, PXR recognizes a number of response elements including DR-3, DR-4, ER-6, ER-8 and IR-0[147]. Binding of the PXR-RXR complex to a response element initiates chromatin remodeling, resulting in the initiation of transcription[148].

5.4. Induction of CYP enzyme expression by PXR

In hepatic tissue, the major CYP isoform upregulated by PXR is CYP3A4, which is estimated to be responsible for the metabolism of approximately 60% of pharmaceuticals[149]. Consequently, PXR activity is of particular clinical importance relative to its role in the mediation of drug-drug interactions[9]. Another CYP enzyme which contains PXR response elements and which can be upregulated by PXR is CYP7A1[147], the enzyme which provides the rate limiting step for bile acid synthesis. Regulation of CYP7A1 expression by PXR is determined by PXR activation by bile acids, thus allowing PXR to also play a major role in the maintenance of bile acid homeostasis[8]. Interestingly, PXR response elements have also been identified in the promoter regions of a number of CYP epoxygenases such as CYP2B6[150] and some CYP2C8/9 [151], CYP2C9[152, 153], and CYP2C19[154]. Furthermore, expression of CYP 2C8/9 has been shown to be upregulated by PXR[155]. The functional significance of PXR's ability to induce the expression of CYP epoxygenases is currently unexplored but may represent an important new component in our understanding of the mechanisms involved in the mediation of vascular tone.

6. Progesterone Synthesis and Metabolism during Pregnancy

Progesterone is a critical hormone for the maintenance of pregnancy in both humans and rodents. In C57BL/6J mice, plasma levels of this 21 carbon steroid hormone rise to ~40 ng/mL within the first few days of pregnancy and remained fixed at this level until approximately day 12[156]. After day 12, progesterone plasma concentration rises rapidly, peaking on day 16 between 80 – 110 ng/mL, and then dropping progressively until parturition[156, 157]. Metabolism of progesterone can be extensive, with enzymatic reduction of double bond between C4 and C5 as well as at the keto moieties at C3 or C20 producing a variety of α and β stereoisomer metabolites. Of particular relevance to this project are the effects of reduction by the 5β-reductase enzyme, which metabolizes progesterone to 5β-dihydroprogesterone, since this molecule is one of the most potent endogenous ligands for PXR.

7. Hypothesis

Considering therefore that pregnancy is a time of both altered vascular function and increased progesterone synthesis/metabolism, that progesterone metabolites are potent activators of PXR, and that PXR can induce the expression of CYP epoxygenases which are capable of producing vasodilatory eicosanoids, we ***hypothesize*** *that PXR contributes to the regulation of vascular tone during pregnancy by enhancing CYP mediated relaxation.*

8. Aims

Using the PXR knockout mouse as a model for this research, we aimed to:

a. Investigate the role of PXR in the mediation of both vasoconstriction and vasorelaxation during pregnancy.

b. Determine the involvement of CYPs and EETs in the signaling pathway of PXR mediated vascular regulation.

c. Examine the expression of PXR and *Cyp2c29* mRNA in mouse mesenteric arteries.

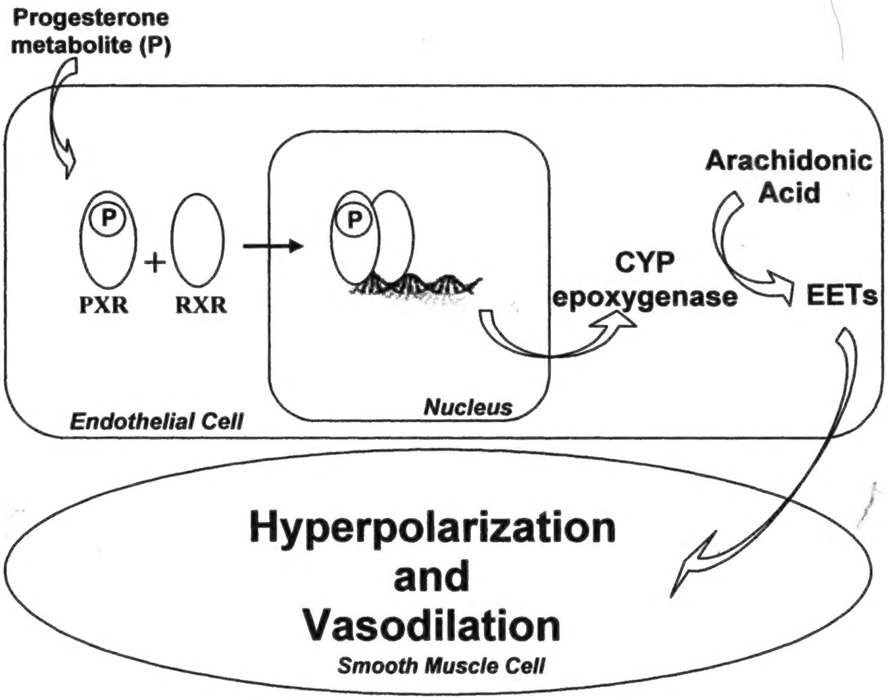


Figure 1 **Schematic representing how progesterone metabolites might act through PXR to induce vasodilation.**

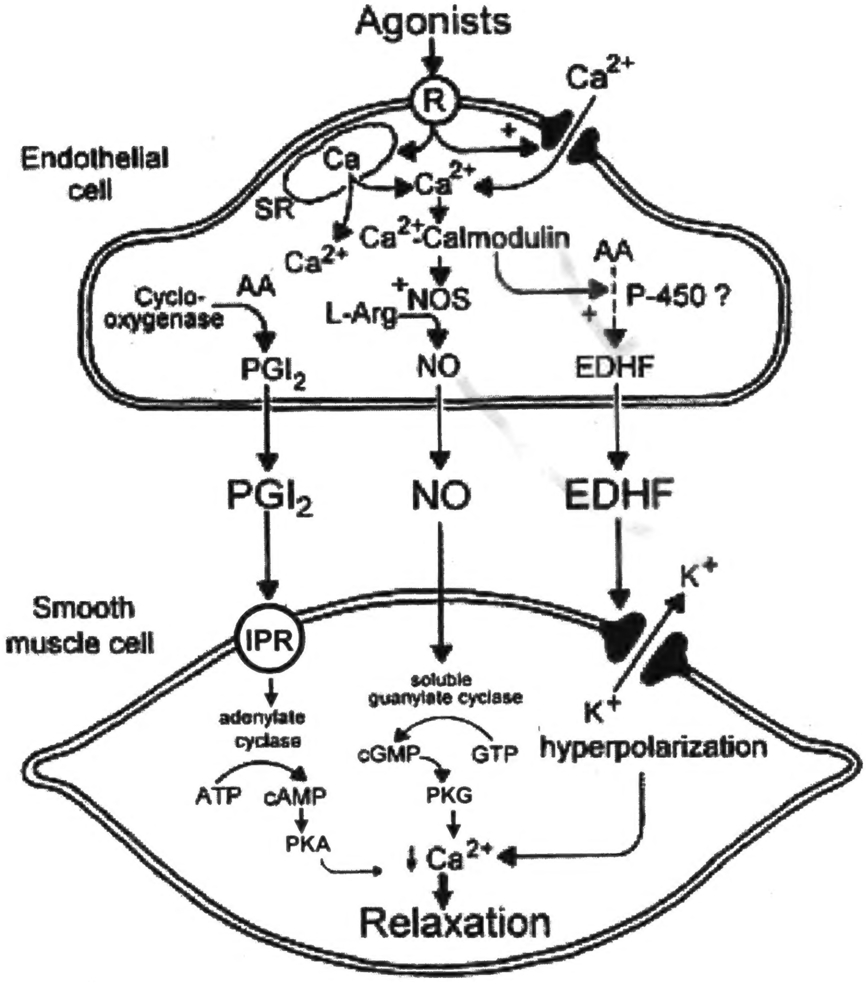


Figure 2 **Endothelium derived relaxing factors act via different pathways to hyperpolarize vascular smooth muscle**

PGI2 and NO induced vascular smooth muscle relaxation primarily through adenylate cyclase and guanylate cycles dependent mechanisms respectively. PGI_2 and NO independent relaxation, here identified as EDHF, induces vascular smooth muscle relaxation via vascular smooth muscle hyperpolarization. PKA = protein kinase A, PKG = protein kinase G, IPR = IP receptor. From C.M. Boulanger and P.M. Vanhoutte, G-proteins and endothelium-dependent relaxations. *J. Vascul. Res.* **34** (1997), pp. 175–185.

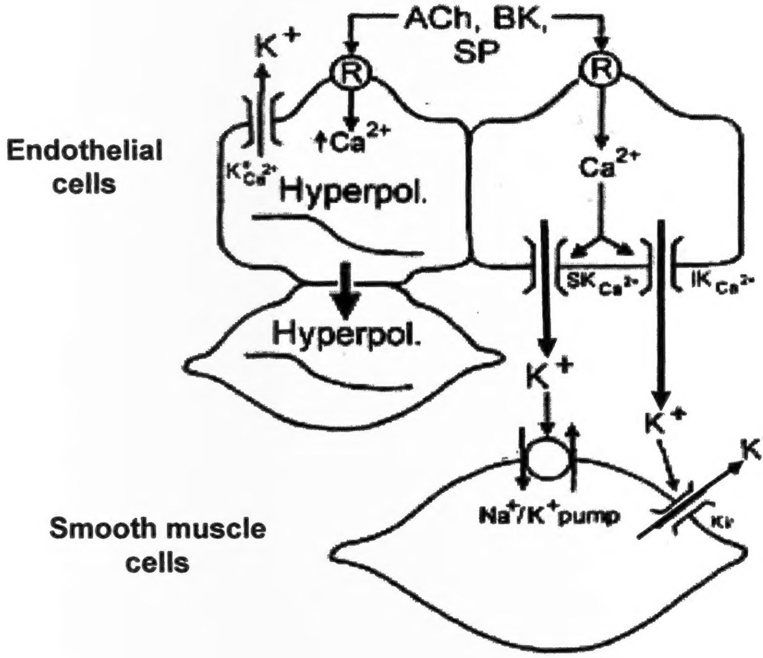


Figure 3 **Mechanisms by which endothelial hyperpolarization induces vascular smooth muscle hyperpolarization**

Activation of SK_{Ca} and IK_{Ca} channels hyperpolarizes the endothelium. Vascular smooth muscle hyperpolarization is then induced either by direct transmission via myoendothelial gap junctions or by the paracrine activation of VSM K_{IR} channels and Na^+-K^+ pumps. Ach = acetylcholine, BK = bradykinin, SP = substance P. From: Vanhoutte PM. Endothelium-dependent hyperpolarizations: The history. *Pharmacol Res*. 2004;49:503-508.

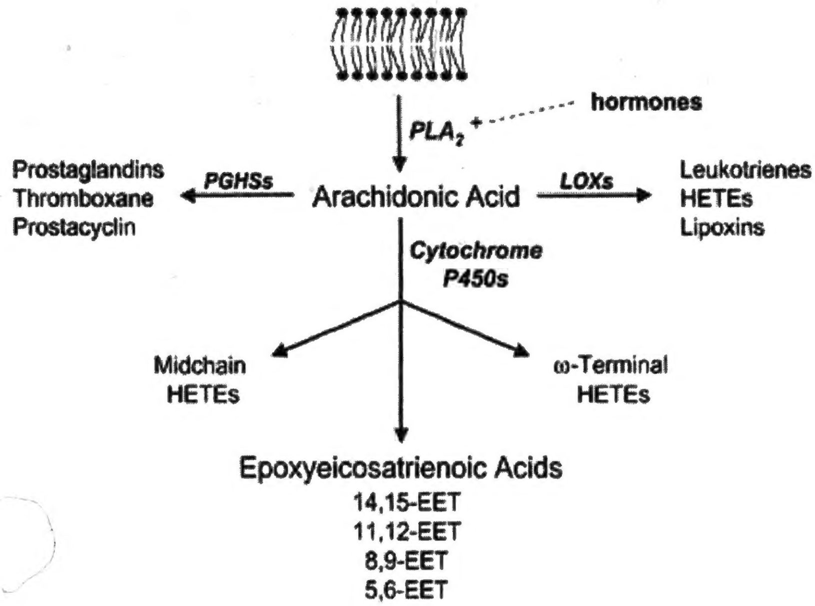


Figure 4 Pathways of arachidonic acid metabolism

Arachidonic acid release from the phospholipid membrane by phospholipase A2 (PLA$_2$) can be metabolized by prostaglandin endoperoxide H synthases (PGHSs), lipoxygenases (LOXs) and cytochrome P450 enzymes. HETEs = hydroxyeicosatetraenoic acids. From: Zeldin, D. C. Epoxygenase pathways of arachidonic acid metabolism *J Biol. Chem.* 2001;276:36059-36062

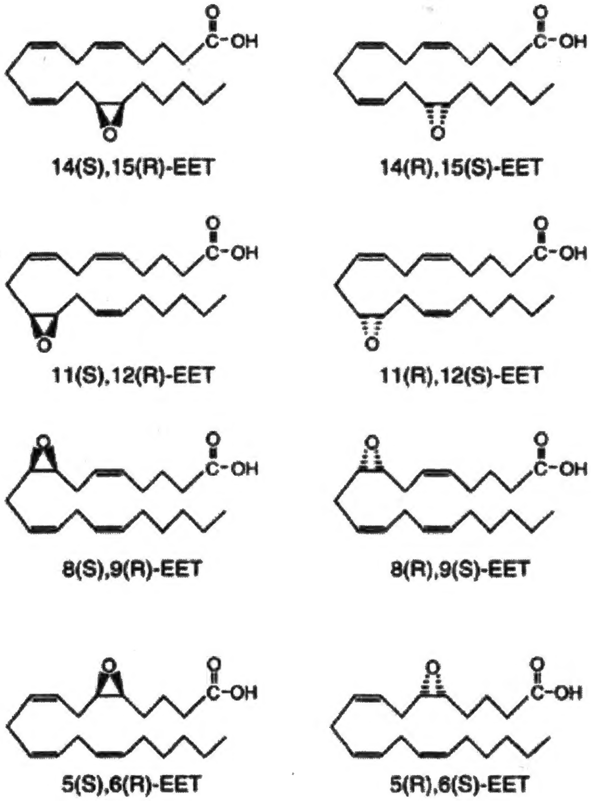


Figure 5 **EET regioisomers**

The four EET regiosiomers can exist in either an S,R conformation or an R, S conformation. From: Zeldin, D. C. Epoxygenase pathways of arachidonic acid metabolism *J Biol. Chem.* 2001;276:36059-36062

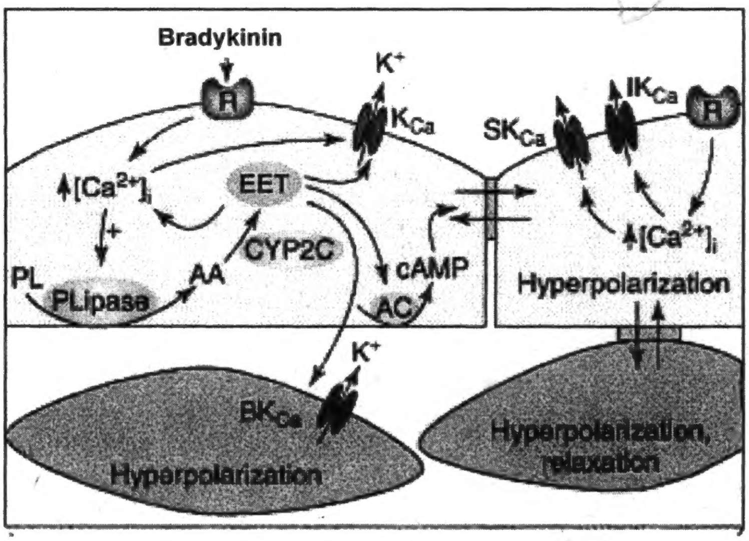


Figure 6 **Mechanisms by which EETs can act as endothelium-derived hyperpolarizing substances**

EETs can act as extracellular messengers by activating BK_{Ca} channels on the vascular smooth muscle or as intracellular messengers by facilitating the rise in intracellular calcium, opening K_{Ca} channels and promoting gap junction communication within the endothelium. From: Busse R, Edwards G, Feletou M, Fleming I, Vanhoutte PM, Weston AH. EDHF: Bringing the concepts together. *Trends Pharmacol Sci.* 2002;23:374-380.

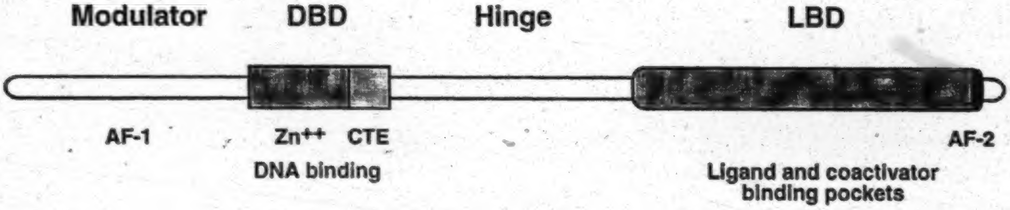


Figure 7 **Structural characteristics of the PXR protein**

The DNA binding domain of all nuclear receptors contains two zinc fingers and a carboxy terminal extension (CTE). Also shown on this diagram are two transcription activation functions (AF-1 and -2)) located at the amino and carboxy termini. DBD = DNA binding domain, LBD = ligand binding domain. From: Giguere V. Orphan nuclear receptors: From gene to function. *Endocr Rev*. 1999;20:689-725.

CHAPTER II - MATERIALS AND METHODS

1. Introduction

The investigation into the role of PXR in the regulation of blood pressure during pregnancy was begun in the spring of 2003 by Dr. C. Cooke, using a new animal model, the PXR knockout mouse, and a preexisting wire myography technique for functional analysis. The preliminary results were promising and warranted further study, including a detailed functional investigation of not only the effect of PXR loss on vascular adaptations to pregnancy, but also the involvement of potential signaling molecules mediating this effect. Further, we also wished to use the highly sensitive technique of real time RT-PCR to characterize the expression profiles of both PXR and CYP molecules in the vasculature of pregnant and non-pregnant PXR wildtype and knockout mice.

Since the preliminary myography data was obtained using a technique that had been developed for another study involving a different mouse model, my initial focus was to optimize the wire myograph protocol for use in this project. Troubleshooting and optimization of the new protocol, including the determining which buffers and reagents to use, running the necessary controls and developing the parameters for assessment of bradykinin induced relaxation comprised a significant portion of the first year of work on this project. Following the collection of the myography data, I worked to develop a method of extracting, storing and quantifying RNA from mouse mesenteric arteries which could be used to provide an RNA template of sufficient quality and quantity to perform real time RT-PCR. The optimization of this protocol was done with the assistance of Dr. Sheena Fang and was part of two undergraduate student projects. Described in the following sections are both the final experimental protocols used and a discussion of their optimization.

2. Experimental Model: The PXR knockout mouse

C57BL/6 mice lacking a functional PXR gene (PXR-/-) and their intact counterparts (PXR+/+) were chosen as the model for this project in order to directly assess the contributions of PXR to pregnancy induced modifications of vascular tone. In 2001, Staudinger et al. generated a PXR -/- mouse by replacing the first coding exon of the

PXR gene, which contains both the translation start site and the first zinc finger of the DNA-binding domain, with a neomycin resistance cassette[158]. This mutation, therefore, results in the production of an aberrant, truncated PXR transcript and further ensures the loss of PXR function by incapacitating the DNA binding domain. Briefly, this was done by isolating a ~15.0-kb fragment containing the first coding exon from the mouse PXR locus and inserting into it a targeting vector with a neomycin phosphotransferase gene under the control of the phosphoglycerate kinase. The mutated plasmid was then cultured and electroporated into mouse embryonic stem cells, allowing for integration of the plasmid into the endogenous PXR locus by homologous recombination. Southern blotting was used to screen for cells containing the mutated locus. Those that were positive were introduced into C57BL/6 blastocysts to generate chimeric males. Ultimately, the chimeric males were bred to C57BL/6 females and the heterozygous offspring were cross bred to produce animals homozygous for the PXR mutation.

3. Functional Analysis

Isometric wire myography was pioneered for use with small arteries in 1977 by Mulvany and Halpern and has been used extensively by a number of researchers to investigate vascular contraction and relaxation responses[159]. Briefly, a vessel is threaded with two smooth wires which are then each tied to an opposing foot of the myograph such that the vessel is suspended between the feet (figure 8). One foot is mobile and can be moved to separate the wires, stretching the vessel and placing tension on its walls in an approximation of the stretch it would receive in vivo. The other foot is stationary and is attached to a force transducer which records the tension generated by the vessel in response to vasoconstricting or vasorelaxing agonists. Typically, a wire myography experiment to assess both constriction and relaxation responses is done in three stages: 1) *mounting*, where the vessel is harvested, mounted and prepared for experimentation, 2) *assessment of constriction* and 3) *assessment of relaxation*. The following section describes the wire myography protocol used in this project and is followed by a section which discusses a number of aspects of the protocol which were optimized specifically for this study.

3.1. Wire myography protocol for mouse mesenteric arteries

3.1.1. Animals and Breeding

C57BL/6J PXR+/+ and PXR-/- mice[158] were generously provided by Dr. B. Goodwin, GlaxoSmithKline, Research Triangle Park, NC. PXR+/+ and PXR-/- females (10-14 weeks old) were time-mated with PXR+/+ males. The observation of a seminal plug was counted as day 0, and pregnant animals were sacrificed on day 17 or day 18 of gestation (term = day 19). Age matched non-pregnant PXR+/+ and PXR-/- littermates were used as controls. All procedures were approved by the University of Alberta Health Sciences Animal Policy and Welfare Committee.

3.1.2. Analysis of pregnancy induced changes in vessel function

Pregnant PXR+/+ and PXR-/- mice (12-16 weeks old) and age matched non-pregnant controls were euthanized by cervical dislocation. Second order mesenteric arteries (100-200 μm in diameter) were carefully dissected free of fat and connective tissue. Dissection was done on ice under a dissecting microscope. Vessels were then mounted on an isometric wire myograph apparatus (Kent Scientific, Torrington, CT) and submerged in 5 mL of warmed Dulbeco's Modified Eagle's Medium (DMEM; Sigma, St. Louis, Mo), which had been prepared on the day of experimentation and was supplemented with 5.5 mmol/L glucose, 1 mmol/L sodium pyruvate and 25 mmol/L HEPES, and pH adjusted to 7.4 at 37°C. After mounting, the vessels were allowed to equilibrate for 30 minutes and a passive circumference-tension curve was performed from which the optimal resting tension was determined using the law of Laplace and an internal pressure of 50mmHg. Vessels were set to the calculated optimum resting tension and equilibrated for another 30 minutes. A phenylephrine (PE) dose response curve (5×10^{-8} – 1×10^{-4} mol/L) was performed first to assess vasoconstriction. Vessels were then washed three times for 10 minutes and the concentration of PE which produced 80% of the maximal response was determined (EC_{80}). This dose was then used to preconstrict the vessels and a second dose-response curve was performed using the endothelial dependent relaxing agent, bradykinin (BK) (10^{-12} – 10^{-5} mol/L) to assess vasorelaxation.

3.1.3. Investigation of the contributions of CYP epoxygenases and EETs to vasoconstriction responses

In a separate series of experiments, three pharmacological agents, N-methylsulphonyl-6-(2-propargyloxyphenyl) hexanamide (MS-PPOH; 50 μM), a non-specific inhibitor of CYP expoxygenases[160], Sulfaphenazole (SUL; 20 μM), a specific inhibitor of CYP2C9 (mouse Cyp2c37), (Sigma)[161], and 14,15-EEZE (10 μM), an EET antagonist (Falck)[162] were used to help elucidate the influence of CYP epoxygenases and 14,15-EET on vascular tone. Vessels were mounted as described above. After the passive curve, vessels were equilibrated for 30 minutes, randomly assigned treatment with either MS-PPOH, SUL or 14,15-EEZE and then incubated with that compound for 15 minutes prior to the start of the PE curve. Once the curve was completed, vessels were washed for ten minutes twice and then exposed to a high potassium-physiological saline solution to assess vessel viability.

3.1.4. Reagents and Solutions

All pharmacological agents were prepared as stock solutions and stored as aliquots at -20°C. PE and BK stocks were dissolved in double distilled water to a concentration of 10mM and were serially diluted in DMEM for each experiment. Inhibitors were dissolved in 100% ethanol at stock concentrations calculated to ensure that the volume of ethanol in the bath never exceeded 0.1% of the total bath volume.

3.1.5. Data Analysis and Statistics

The mean PE EC_{50} was used to summarize all PE dose response curves and two-way analysis of variance (ANOVA) with least significant differences post hoc testing was used to compare vasoconstriction responses. Relaxation curves were analyzed using two-way ANOVA with repeated measures. All data are expressed as mean ± standard error of the mean (SEM) and statistical significance was set at $p<0.05$.

3.2. Optimization of the wire myograpy protocol

3.2.1. Alterations to the preexisting protocol

The wire myography protocol used was based on that developed previously in our laboratory by Dr. C. Cooke[163]. However, a number of modifications were made to further optimize the protocol specifically for these studies as follows:

- Although physiological saline solution is traditionally used for myography, Dr. Cooke determined that a more supportive media such as Dulbecco's Modified Eagles Medium (DMEM) was beneficial when working with mouse vessels. For these experiments, DMEM was still used, however, the concentration of HEPES was increased from 5 mM to 25 mM to improve the buffering capacity of the media.

- Bradykinin was used as the endothelial dependent relaxing agent (as opposed to methylcholine) since in this model, BK induced vasorelaxation proved to be greater and more reliable than that induced by methacholine. Further, since activation of the BK B_2 receptors is known to cause cleavage of arachadonic acid from the plasma membrane[164], it was felt that from a theoretical perspective BK was also a superior agent since its use would ensure an available supply of substrate for CYP metabolism. Moreover, there is evidence to suggest that BK is more likely to stimulate the release EDHF than acetylcholine[164].

- Both the PE and BK dose response curves were tailored to this model such that the first dose was a lower concentration than was previously used and there were more doses in smaller increments in the linear phase of the response. These changes created a sigmoidal shaped curve in which the first point had a 0% response and increased the number of data points in the linear portion of the curve, thereby improving the repeatability of the data and the accuracy of data analysis.

Interestingly, in our studies, the amount of relaxation of observed in the mesenteric arteries of female mice preconstricted with PE and stimulated with BK was considerably less that the amount of relaxation reported by others conducting similar experiments. In our studies, relaxation in vessels from non-pregnant mice (either PXR+/+ or PXR-/-) was approximately 30% of the amount of PE preconstriction. In contrast, both Myers et al[165]. and Longo et al.[166] assessed relaxation in mesenteric arteries from C57BL/6 non-pregnant female mice preconstricted with PE and found acetylcholine induced relaxation in these vessels to be approximately 80%. Although relaxation was stimulated with a different endothelial dependent relaxing agent this is unlikely to account for a difference of almost 50% in the relaxation responses. One possible explanation is related to the use of DMEM as opposed to physiological saline solution as media for these experiments. Although DMEM is a more supportive media than physiological saline solution, difficulties associated with maintaining pH required increasing the HEPES content of the buffer to 25 mM. Unbeknownst to us at the time was the fact that HEPES concentrations greater than 10 mM can inhibit gap junctions[167]. As has been previously discussed, inhibition of gap junctions could possibly interfere with EDHF dependent relaxation. However, although unintentional, inhibition of gap junctions was advantageous as it removed the endothelium-derived hyperpolarization portion of the EDHF response and allowed for the investigation of relaxation due solely to the release of relaxing factors from the endothelium.

3.2.2. Age of animals used for experimentation

Previous members of the Davidge laboratory determined that animals aged 12-16 weeks were optimal for use in wire myography studies since animals of this age had vessels that were slightly larger and therefore were more suited to myography studies than younger animals. Due however, to an unfortunate mix up in the animal colony, the only animals available for use when these experiments were started were between 27 and 33 weeks of age. Data were collected from 2-4 animals in each group before younger animals were available. Because age can affect vessel function we compared the data collected from older animals with preliminary data collected from younger animals (figure 9) to determine if we could combine the data or not. Although vascular function in the older animals reflected the same trends between groups as was seen in the younger animals, it was decided to not combine the data since the results from these first few experiments were highly variable and there was a notable difference in maximum relaxation between the old and young animals in the non-pregnant PXR-/- group.

3.2.3. Serial curve repeatability

When planning the inhibitor experiments, we wanted to use a paired design, since this greatly increases the power of analysis. To do so, vessel function is first assessed in the absence of the inhibitor, then vessels are washed for 30 minutes, incubated with the inhibitor and the curve is repeated. This style of experimental design requires there to be no difference in vessel function due to the passage of time or because of prior preconstriction/exposure to BK. Therefore, in preparation for these experiments, we performed a series of control experiments in which two curves were conducted sequentially without any inhibitor in order to assess the repeatability of the curves. Since we were most interested in using the inhibitors to investigate the role of CYPs and EETs in relaxation, we began by assessing the repeatability of the BK curve. Vessel function data from three non-pregnant PXR+/+ mice were averaged for each of the first and second curves and the maximum relaxation of each curve was then compared by paired t-test. We found that there was a strong trend for the second curve to relax less to a given dose of BK than the first curve (max relaxation first curve: 29 ± 2% vs. second curve 20 ± 1%, $p=0.057$; figure 10). Since the first and second curves were not identical, we could not use a paired design experiment. It was therefore necessary to run two separate but simultaneous experiments to assess the effect of the presence or absence of the inhibitor on vessel function. This is the protocol that was used to test the effects of the inhibitors on PE-induced constriction. Ultimately, however, as is discussed at the end of the following section, additional difficulties associated with the nature of the BK curve in pregnant animals further prevented the use of inhibitors to assess CYP/EET involvement in the vasorelaxation response.

3.2.4. Time controls

To confirm that the observed relaxation response was due activation of the endothelium by BK rather than a result of spontaneous relaxation, time controls were performed to ascertain the stability of the PE induced preconstriction plateau. To do so, vessels were mounted, and a PE curve was performed. Vessels were then preconstricted, and vessel tension was recorded every 2.5 minutes for at least 10 minutes, which is the time required to complete the BK dose response curve.

Data from time control experiments showed that, in response to a bolus dose of PE, mouse mesenteric arteries describe a characteristic pattern of constriction such that in the first 30 seconds there is a sharp, spike like rise in tension (point A on figure 11) which quickly drops to approximately 50 - 75% the height of the spike (point B). For the next 5 – 7 minutes, tension gradually rises, reaching a maximum (point C) and falling again, eventually plateauing (point D) after approximately 10 minutes. In healthy vessels, this plateau was then stable for the remainder of the time recorded. Thus, for experimentation, a preconstriction period of 15 minutes was introduced to allow for a stable plateau to be reached before the first dose of BK was given. Over the course of experimentation, however, it became evident that not all vessels prepared for myography were able to achieve a stable plateau. Thus, based on the responses of vessels from all four treatment groups, a set of inclusion criteria were developed and applied retrospectively to select vessels for analysis. The inclusion criteria stated that in order to be included for analysis a vessel must have achieved a preconstriction plateau of at least 0.09 g of tension above baseline and must have held the preconstriction plateau for at least 90 seconds. This ensured we analyzed only vessels in which the response observed was in fact the result of BK stimulation and not due to spontaneous relaxation.

Interestingly, the ability of a vessel to maintain preconstriction varied depending on type of vessel under investigation. Specifically, vessels from pregnant animals (either PXR +/+ or PXR -/-) were much less likely to hold a plateau than their non-pregnant counterparts. On average, only 36% of vessels from pregnant animals that were mounted maintained preconstriction and were able to be used for assessment of relaxation whereas, in vessels from non-pregnant animals a mean of 70% of the vessels mounted held a plateau and could be used to test relaxation. While this tendency for vessels from pregnant animals to spontaneously relax was not surprising given the vascular adaptations which occur during pregnancy, it required a large number of animals to be sacrificed to collect a sufficient amount of data for analysis. Moreover, it also prevented the investigation of CYP and EET involvement in the mediation of vasorelaxation during pregnancy because our protocol required the inhibitors to be applied before preconstriction, yet we could not know prior to preconstriction which vessels would maintain a suitable preconstriction plateau. Therefore, analysis of the contribution of CYP epoxygenases and EETs to vascular function was limited to an investigation of their roles in the mediation of PE induced vasoconstriction during pregnancy.

3.2.5. Choice of inhibitors

A variety of agents are available to inhibit CYP epoxygenase activity, each of which has advantages and disadvantages for use. Although imidazole compounds such as clotrimazole and miconazole are frequently used as CYP epoxygenase inhibitors, they have been reported to also directly inhibit Ca^{++}-dependent K^+ channels[168]. The acetylenic compound, MS-PPOH, acts as a suicide substrate inhibitor, binding to the enzyme and permanently inactivating it. It functions to block the activity of all CYP epoxygenases but does not inhibit CYP ω-hydroxylation[160] and is not known to affect K^+ channel function, making it a good tool to assess global CYP epoxygenase function. The IC_{50} of MS-PPOH was determined by Wang et al. to be 13 μM. However, we chose to use a concentration of 50 μM, which inhibits renal epoxygenase activity by >80% and which has been shown to be more efficacious than 5 μM MS-PPOH in altering the renal afferent arteriolar response to increased perfusion pressure[77]. Treatment with 50 μM MS-PPOH has also been shown to attenuate the nitric oxide-independent component of vasorelaxation elicited by BK in renal afferent arterioles[93]. One limitation to using this concentration of MS-PPOH is that it has been shown to inhibit COX activity by up to 40%[160]. However, as previously noted, COX prostaglandins do not contribute significantly to vascular tone during pregnancy. Furthermore, since we hypothesized CYP regulation of vascular tone is enhanced during pregnancy, we still chose to use this concentration of MS-PPOH in order to ensure that we were using a concentration of inhibitor which would be effective in all treatment groups.

The use of the broad specificity epoxygenase inhibitor, MS-PPOH, allowed us to determine if changes in the vascular response were due to the activity of any CYP epoxygenase isoform. However, because CYP2C8/9 has been implicated in the mediation of the EDHF response in coronary arteries, we also wanted to determine if this isoform was playing a similar role in mouse mesenteric arteries. Therefore, the specific CYP2C9 inhibitor, SUL, was chosen to complement the data collected using MS-PPOH. SUL is a reversible inhibitor which remains specific for CYP2C9 (mouse Cyp2c37) even at concentrations as high as 100 μM[161]. It is also capable of inhibiting CYP2C8 (mouse Cyp2c29) although it is much less potent[169]. Finally, we also used the EET antagonist, 14,15-EEZE to asses the contribution of EETs to PE induced vasoconstriction. 14,15-EEZE is a 14,15-EET analogue which can inhibit vasorelaxation induced by all EET isomers. However, it is most effective at inhibiting 14,15-EET activity[162]. Since, we do not know which EET isomer may be involved, the use of this general inhibitor is advantageous in these investigations. Together, the use of a broad specificity CYP epoxygenase inhibitor, a specific CYP inhibitor and an EET inhibitor will provide a comprehensive picture of the role of CYPs and EETs in the mediation of PE induced vasoconstriction in non-pregnant and pregnant states.

4. Molecular Biology

We hypothesized that PXR mediates vascular changes during pregnancy by enhancing the expression of CYP expoxygenases. Therefore, in addition to functional analysis, we also used quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis to examine the effect of pregnancy on PXR and CYP mRNA levels in the mesenteric arteries of PXR +/+ and PXR -/- mice. There are a variety of chemistries available for use in qRT-PCR. We used Taqman primer and probe sets, which employ a probe conjugated with a fluorescent dye to report the amount of amplicon formed as the PCR reaction progresses, thereby giving a real time assessment of the PCR reaction. As a result, amplicon production can be measured during the exponential phase of the PCR reaction when the amount of PCR product is directly proportional to the initial amount of target template. This makes qRT-PCR a highly sensitive means of determining gene expression and, most importantly, allows for accurate quantitation and comparison of gene expression between treatment groups. Moreover, qRT-PCR is also highly specific because not only are the primers designed to detect only the target gene, but also the reporter probe is designed to hybridize only to the gene of interest, thereby further limiting the possibility of non-specific amplification. This combination of high sensitivity and high specificity made qRT-PCR ideal for our purposes. However, before we could proceed with qRT-PCR analysis of gene expression we first had to develop a protocol to obtain RNA from mouse mesenteric arteries. Detailed in the following sections is a discussion of 1) the development of an optimized protocol to collect mouse mesenteric arteries, extract their RNA, and analyze it for quality and quantity and 2) the choice and optimization of primer/probe sets for use in this project. The final experimental protocol for qRT-PCR analysis is described in section 3.3.

4.1. Development of a protocol for RNA extraction

Preliminary work preformed by the Perinatal Research Centre core technician suggested that extraction of RNA from mouse mesenteric arteries was possible. However both the quality and quantity of the RNA obtained was limited. Consultation with technicians from Dr. P. Halloran's laboratory, Division of Medical

Microbiology and Immunology, Department of Medicine, University of Alberta, the Perinatal Research Analysis Core, and the Qiagen technical support centre, identified four main areas of concern within the existing protocol which might contribute to possible RNA degradation or loss including: length of time of dissection, incomplete homogenization of the tissue, method of storage of harvested tissue and length of time that tissue was stored before use. Based on recommendations from these groups, it was decided that modified versions of either the classic Trizol extraction method or the Qiagen RNeasy micro kit (Qiagen, Mississauga ON) would be the most likely to produce useable RNA. Therefore, a panel of experiments was designed (Table 1) to test a variety of tissue harvest and extraction techniques in an effort to obtain RNA from mouse mesenteric arteries of sufficient quality and quantity to be able to perform at least 3 real-time RT-PCR reactions and corresponding negative controls.

All animals used for these optimization experiments were non-pregnant PXR +/+ females, aged 12-14 weeks. The harvest and dissection protocols were based on those used for the myography experiments. However, to decrease the risk of RNA degradation, some changes were made to minimize the amount of time between the death of the animal and the time when the vessels were harvested. Specifically:

- a stock of dissection media for all experiments was made up ahead of time, filtered through a 0.22 μl filter, pH adjusted to 7.4 at 4.0°C to decrease the amount of time needed to set up for an experiment and to sterilize the media
- the intestines and mesenteric arcade were harvested immediately into ice cold DMEM but no other tissues were collected
- once a vessel was dissected free from the surrounding tissue, it was placed immediately into either a labeled, 2.0 mL RNAse free microcentrifuge tube which was pre-frozen and stored in a cooler of liquid nitrogen on the bench for the duration of the dissection (Trizol method) or into a labeled, 2.0 mL RNase free microcentrifuge tube filled with buffer RLT (containing guanidinium salts and β-mercaptoethanol to inhibit RNAses) from the RNeasy micro kit which was kept on ice for the duration of the dissection. By freezing the tissues or placing them in a RNAse inhibiting solution immediately upon harvest we hoped to decrease

the risk of RNA degradation associated with the dissection. On average, the amount of time between sacrifice and the collection of the last vessel was under 2 hours.

4.1.1. *RNA extraction using the modified Trizol method*

For the modified Trizol method, microcentrifuge tubes containing the frozen arteries were collected from the -80°C freezer and put on ice. 350 µl of Trizol (Invitrogen, Burlington, ON) was dispensed into each tube and the arteries were homogenized immediately for 2 x 20 seconds using PRO200 homogenizer with a 5 mm Generator (DiaMed Lab Supplies INC, Mississauga ON). After homogenization, a 1:5 volume (~70 µl) of chloroform (Fisher, Nepean ON) was added, tubes were inverted 20 times, and then centrifuged at high speed for 15 minutes at 4°C. Following centrifugation, the aqueous layer was removed to a new RNase free microcentrifuge tube and 1 volume of isopropanol (~300 µl) was added to precipitate the RNA. Additionally, a 1:10 volume (~30 µl) of 2.0 M sodium acetate, pH = 4.0 (Sigma, St. Louis MO) and 20mg molecular grade glycogen (Roche, Mississauga, ON) were also added to promote RNA precipitation and pellet visibility and the tube was placed at -80°C for 72 hours to precipitate the RNA. Following precipitation, the tube was centrifuged at high speed for 30 minutes at 4°C to pellet the RNA. The supernatant was then removed and 400 µl 75% ethanol was added. The tube was inverted to wash the pellet then centrifuged again at low speed for 5 minutes at 4°C. Once the pellet was washed, the ethanol was removed and the pellet allowed to air dry. The purified RNA was then resuspended in 10 µl RNAse free water, 2.0 µl was removed for analysis of quality and quantity, and the remaining 8.0 µl was stored at -80°C.

4.1.2. *RNA extraction using the Qiagen RNeasy micro kit*

For Qiagen RNeasy micro kit method, RNA extraction was performed according to the manufacturer's specified protocol with minor modifications. Specifically, tubes of frozen arteries were removed from the freezer and kept on ice. 350 µl of Buffer RLT supplemented with 3% β-mercaptoethanol and 20 ng of carrier RNA were added to the tubes and the arteries were homogenized for 2 bursts of 20 seconds. The sample was then further homogenized by placing on a QIAshredder Spin Column (Qiagen, Mississauga, ON) and centrifuging at maximum speed for 2 minutes. Following homogenization, the homogenate was centrifuged for 3 minutes at maximum to pellet any debris. Once centrifugation was complete, the supernatant was removed to a new tube, 350 µl of 70% ethanol was added, the entire sample was applied to an RNeasy MinElute Spin Column and the column was spun for 15 seconds at 10,000 rpm. The column was then washed by adding 350 µl Buffer RW1 and spinning for 15 seconds at 10,000 rpm. Following the wash step, the sample was incubated with 10 µl (27 Kunitz Units) DNase 1 (Qiagen, Mississauga, ON) for 15 minutes at room temperature. After DNAse treatment, the column was washed sequentially with 350 µl Buffer RW1, 500 µl Buffer RPE and 500 µl 80% ethanol. Finally, the RNA was eluted from the column with 14 µl of RNase free water (Invitrogen, Burlington, ON). 2.0 µl of purified RNA were reserved for analysis of quality and quantity and the remainder of the RNA was stored at -80°C.

4.1.3. Analysis of RNA quality and quantity

The extraction of RNA using the modified Trizol and Qiagen kit methods was coordinated such that all RNA samples were ready for analysis on the same day. All samples were kept at -20°C upon extraction and were submitted to University of Alberta's Institute for Biomolecular Design (IBD) for analysis within 4 hours of collection. The IBD uses an Agilent 2100 Bioanalyzer with the RNA 6000 Nano LabChip Kit to determine the quality and quantity the RNA sample. The Agilent 2100 bioanalyzer employs a microfluidics-based platform to accurately quantify as little as 5 ng/µl of total RNA. Further, it also gives a standardized assessment of RNA integrity based on analysis of the electrophoretic trace of the RNA sample, and detection of the presence or absence degradation products. This provides a more accurate assessment than 28S:18S ratio because it looks at the whole electrophoretic trace rather than just two bands. The quality of the RNA sample is indicated by the RNA Integrity Number (RIN) which can then be used to determine whether or not to use the sample in downstream applications. The RIN is a numeric scale from 1 to 10, with 1 being the most degraded and 10 being the most intact. Although each lab must establish its own quality threshold, general guidelines suggest that a RIN >7.0 is sufficient for use in PCR applications (personal communication, Anna Hutton at IBD and Halloran lab technicians).

4.1.4. Outcome of RNA analysis

The results of the Agilent analysis of RNA quality and quantity are shown in table 2. RNA was successfully extracted from all but two of the samples, both of which were from kit extractions, although from different treatment groups. There was no immediately evident explanation for why RNA was not collected from these samples, although the most likely explanation was that the extracted RNA failed to elute from the column in these cases.

It is difficult to directly compare the RNA yield for each method since the Trizol extraction yields total RNA while the Qiagen kit uses size exclusion principles to yield total RNA *enriched for mRNA* by removing all RNA >200 base pairs. Thus, while the concentration of RNA from the kit appears significantly lower than in those samples extracted with Trizol, if the amount of mRNA in each of the Trizol samples is calculated (based on an estimated 3% mRNA in total RNA) these samples would yield 5.5 ng/μL and 4.0 ng/μL mRNA respectively.

The quality of the RNA extracted using Trizol was high (RIN between 8 and 8.8) and very consistent. The RNA extracted using the RNeasy kit was also of high quality (RIN between 9.5 and 6.5), but it was also quite variable. A RT-PCR test showed all samples to be capable of amplifying the housekeeping gene, hypoxanthine guanine phosphoribosyl transferase.

Overall, these pilot results indicated that it is possible to get RNA of sufficient quality and quantity from mouse mesenteric arteries for use in real time RT-PCR. Although both extraction techniques yielded useable RNA, we opted to continue to use the modified Trizol method since it is easy to perform, does not run the risk of sample loss associated with column extractions, and yields consistently high quality RNA.

4.2. Primers used for qRT-PCR

Commercial taqman reagents, obtained from Applied Biosystems, were used in this project in order to minimize the amount of optimization required. The PXR primer and probe set was chosen because it spanned the boundary between exon 1 and 2 of the PXR gene, thereby allowing us to use this primer to both examine the effect of pregnancy on PXR expression in the PXR +/+ animals and to confirm the PXR -/- genotype. As for analysis of CYP expression, because a number of CYP enzymes could potentially be involved in the regulation of vascular tone during pregnancy, we would have ideally liked to test the expression of as many epoxygenase isoforms as possible. Unfortunately, however, we were limited by RNA availability. We sought to address this difficulty by designing primers which would detect multiple *Cyp2c* genes at once since members of the Cyp2c family are currently the most likely and best studied candidates for involvement in the EDHF response. The only primer set which would detect all of the mouse *Cyp2c* genes would produce an amplicon of ~800 base pairs, a size incompatible with quantitative RT-PCR. Therefore, we choose to assay only the expression of *Cyp2c29*, since it is known to be involved in the EDHF response. Ideally, we would have also chosen to assess *Cyp2c37*, but those primers were not available at the time the experiment was designed. 18S was chosen as the housekeeping gene since its level of expression is not known to be influenced by pregnancy.

Primer efficiencies were determined using the standard curve method. To do so, 5 three fold serial dilutions of liver RNA were prepared and run with each of the primers/probe sets. Efficiency (E) was calculated using the equation $E=10^{(-1/slope)}$ [170]. After excluding any outliers, the efficiencies of the PXR, *Cyp2c29* and 18S primers were determined to be 1.84, 1.77, and 1.86 respectively. While this is slightly lower than the ideal efficiency of 1.90 – 2.00, since the efficiencies were within a 10% range of one another it was elected to proceed using these primers as validated.

4.3. Experimental RT-PCR protocol

Non-pregnant and pregnant PXR+/+ mice were euthanized by overdose of isofluorane (Bimeda-MTC Animal Health Inc., Cambridge ON) and the entire mesenteric arcade was harvested and instantly placed on ice in DMEM. All mesenteric arteries were dissected immediately and snap frozen in liquid nitrogen. Three pools (representing two mice each) of arteries were collected from each treatment group. Arteries were stored at -80°C until all samples had been collected (maximum storage time of ~2 months). RNA was extraction was done in two batches of six samples (containing at least one sample from each treatment group) using Trizol™ (Invitrogen, Burlington, ON). 1 μg glycogen (Roche, Mississauga, ON) and 1:10v/v sodium acetate (2.0 mol/L, pH=4.0; Sigma, St. Louis, Mo) were used to promote RNA precipitation. 500 ng of RNA was reverse transcribed with Superscript II and random primers (Invitrogen, Burlington, ON), aliquoted and stored at -80°C until use. Assays on Demand primers and taqman probes for PXR (catalogue #Mm00803088_m1), *Cyp2c29* (catalogue #Mm00725580_s1) and 18S (cat #4333760T) were obtained from Applied Biosystems (Streetsville, ON) and run in 20 μl reactions according to manufacturer's specifications in an ICycler PCR machine (Bio-Rad, Mississauga, ON). The cycling protocol was as follows: 10 minutes at 95°C, followed by 45 cycles of denaturing for 15 seconds at 95°C and annealing/extension for 1 minute at 60°C. Liver RNA was included as a positive control and no-template and no-RT negative controls were run to verify the PCR results.

4.3.1. Data Analysis

Threshold cycles were automatically determined by the ICycler software. Sample C_T values were normalized to 18S expression, and the ratio of relative expression between samples was calculated using the Pfaffl method[170]. To do so, triplicate values for each sample were averaged to obtain a mean C_T value for each sample for both the 18s and the *CYP2c29* primer sets. One sample was then arbitrarily chosen as the standard against which all other samples were compared to obtain a ΔC_T value. The effect of the efficiency (E) of the PCR reaction was then taken into account using the equation $E^{\Delta CT}$ to calculate values for all samples for both the *2c29* and 18S primer sets. *Cyp2c29* gene expression was then normalized to 18S expression by taking the ratio of *Cyp2c29* expression to 18S expression. Finally, the normalized values of *Cyp2c29* expression were averaged for each treatment group and compared using a t-test.

Tables

Table 1 **Experimental design for optimization of RNA extraction technique**

SAMPLE ID	METHOD OF STORAGE	EXTRACTION TECHNIQUE
KH5-2	Vessels snap frozen on bench	Modified Trizol
KH5-3	Vessels snap frozen on bench	Modified Trizol
KH5-4	Vessels snap frozen on bench	Qiagen RNeasy Micro kit
KH5-5	Vessels snap frozen on bench	Qiagen RNeasy Micro kit
KH5-6	Vessels harvested into buffer RLT, homogenized and snap frozen	Qiagen RNeasy Micro kit
KH5-7	Vessels harvested into buffer RLT, homogenized and snap frozen	Qiagen RNeasy Micro kit
KH5-8	Vessels harvested into buffer RLT RNA extracted immediately	Qiagen RNeasy Micro kit
KH5-9	Vessels harvested into buffer RLT RNA extracted immediately	Qiagen RNeasy Micro kit

Table 2 **Concentration and RNA integrity number for extracted RNA**

SAMPLE ID	CONCENTRATION (ng/μL)	RNA INTEGRITY NUMBER (RIN)
KH5-2	185	8.8
KH5-3	134	8.0
KH5-4	8	8.7
KH5-5	None detected	--
KH5-6	None detected	--
KH5-7	8	9.5
KH5-8	17	7.5
KH5-9	7	6.5

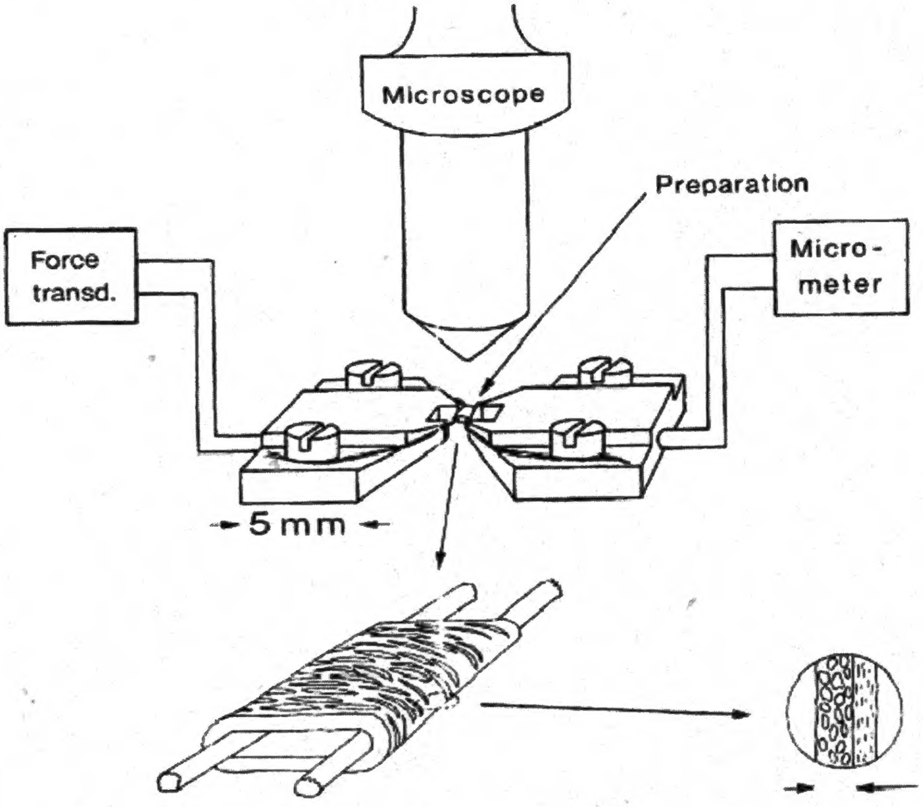
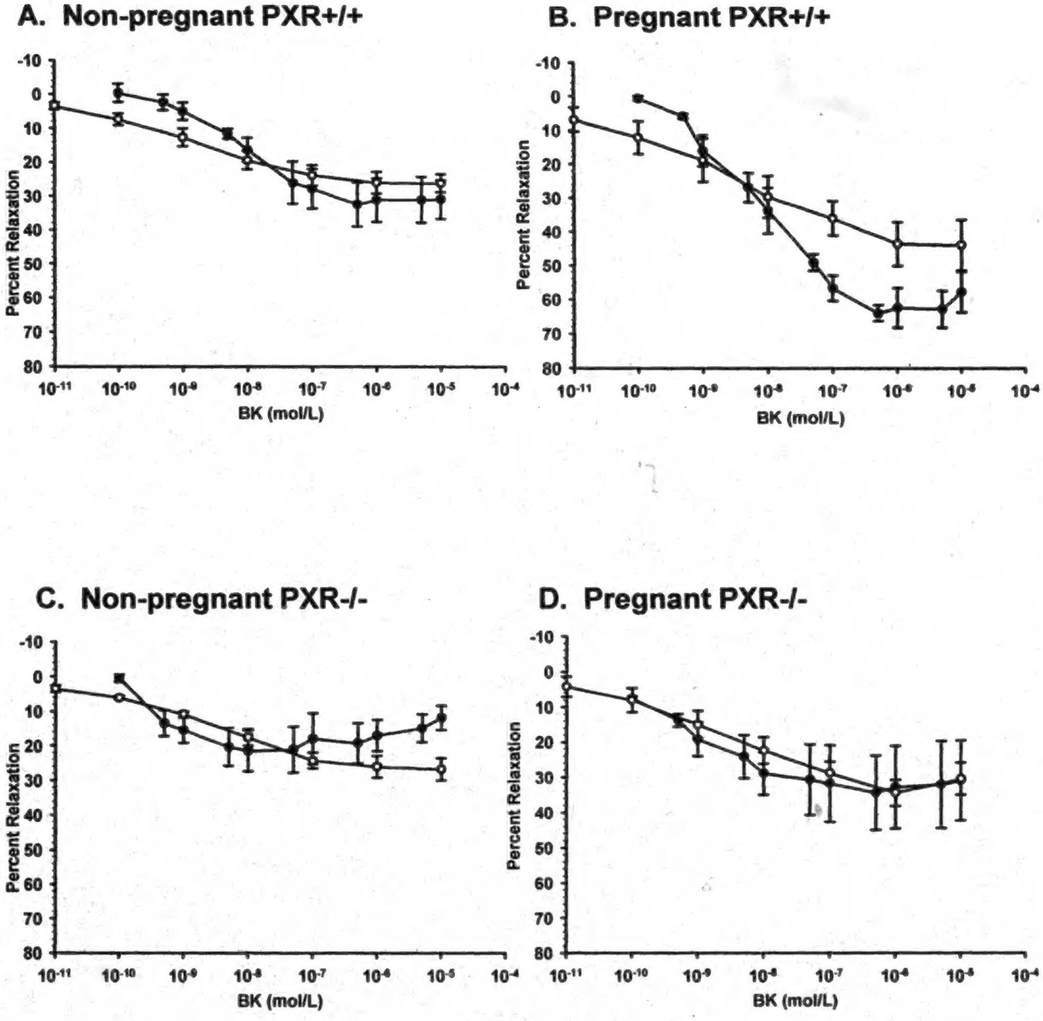


Figure 8 **Diagram of the wire myograph apparatus with mounted artery**

From Mulvany et al. *Physiological Reviews* 1990: 922-949



A. Non-pregnant PXR+/+

B. Pregnant PXR+/+

C. Non-pregnant PXR-/-

D. Pregnant PXR-/-

Figure 9 Comparison of vasorelaxation responses in old and young mice

BK induced relaxation in **A)** old (●, n=3) and young (○, n=5) non-pregnant PXR+/+ mice, **B)** old (●, n=2) and young (○, n=4) pregnant PXR+/+ mice, **C)** old (●, n=4) and young (○, n=3) non-pregnant PXR-/- mice and **D)** old (●, n=2) and young (○, n=3) pregnant PXR-/- mice. Values represent mean ± SEM

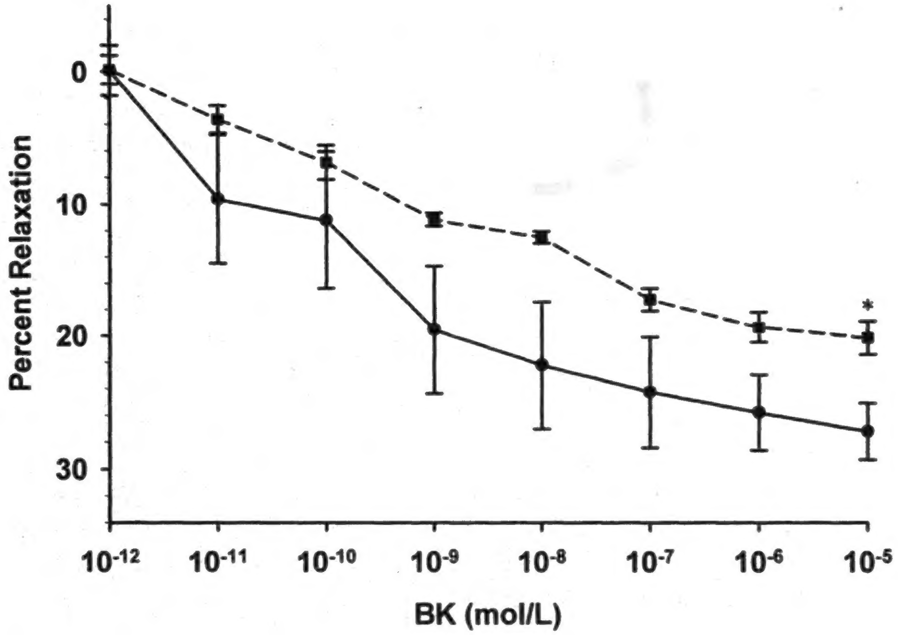


Figure 10 **Comparison of the first and second relaxation responses of vessels serially stimulated with BK**

Maximum relaxation from each of three non-pregnant PXR+/+ mice was averaged for the first (●) and second (■) BK curves and compared using a paired t-test. Values represent mean ± SEM. * $p > 0.05$.

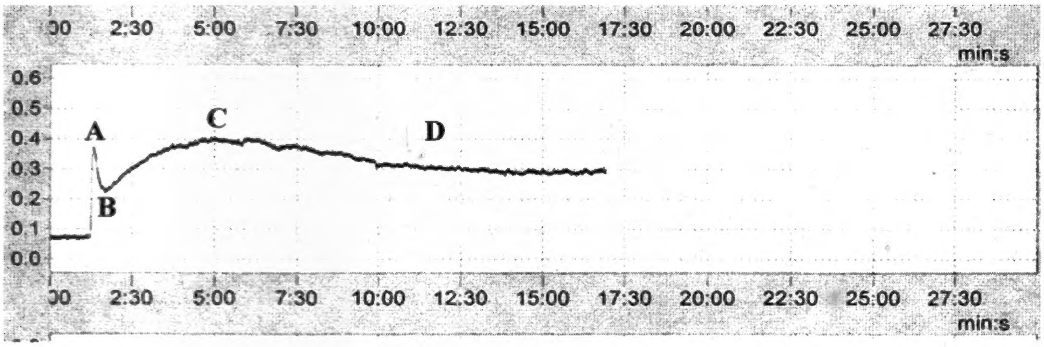


Figure 11 **Representative trace of PE induced preconstriction and plateau in a mesenteric artery from a non-pregnant PXR+/+ mouse**

The initial rise in tension following addition of phenylephrine (**A**) quickly drops to approximately 50 - 75% the height of the spike (**B**). For the next 5 – 7 minutes, tension gradually rises, reaching a maximum (**C**) and falling again, eventually plateauing (**D**) after approximately 10 minutes.

CHAPTER III - RESULTS

1. Phenotypic effects of PXR knockout on gestation and progeny

Loss of PXR function had minimal phenotypic effects on gestation and pregnancy. The average length of gestation was the same between PXR+/+ and PXR-/- mice. There was also no difference in pre-pregnancy weight, average maternal weight gain (adjusted for day of gestation), average fetal pup weight or number of fetal pups/litter between the genotypes. There was, however, a significant difference in the average litter size, with KO animals delivering fewer live pups per litter as compared to their WT counterparts. (PXR+/+ = 7 pups/litter vs. PXR-/- = 5 pups/litter; n=25, 22 respectively, $p<0.001$).

2. Pregnancy induced changes in vascular function in mesenteric arteries from PXR+/+ and PXR-/- mice

In order to directly assess the contributions of PXR to the regulation of vascular tone during gestation we used wire myography to compare the constriction and relaxation responses of small mesenteric arteries from non-pregnant and pregnant PXR+/+ and PXR-/- mice. We found that vessels from PXR+/+ pregnant mice were significantly less sensitive to stimulation with the α_1-adrenergic agonist, PE, than were vessels from non-pregnant PXR+/+ mice were (non-preg EC_{50}: 2.77 ± 0.32 μmol/L vs. preg EC_{50}: 5.13 ± 0.36 μmol/L, p=0.009; figure 12a). In contrast, vessels from pregnant PXR-/- animals did not show this characteristic pregnancy induced reduction in vasoconstriction (non-preg EC_{50}: 3.33 ± 0.38 μmol/L vs. preg EC_{50}: 3.65 ± 0.26 μmol/L, p=0.546; figure 12b). This inability for PXR-/- animals to generate appropriate alterations in vascular function in response to pregnancy is also reflected in the fact that vessels from pregnant PXR-/- mice have a significantly greater vasoconstriction response than vessels from pregnant PXR+/+ mice (PXR+/+ EC_{50}: 5.13 ± 0.36 μM vs PXR-/- EC_{50}: 3.65 ± 0.26 μM, p=0.034).

A similar PXR dependent effect was observed when we assessed the influence of pregnancy on vasorelaxation. Vessels from pregnant PXR+/+ animals demonstrated enhanced relaxation to BK when compared to their non-pregnant counterparts

(ANOVA: p=0.004; figure 13a) but this pregnancy induced adaptation was absent in PXR-/- animals (ANOVA: p=0.806; figure 13b).

3. Contribution of CYP epoxygenases and EETs to vasoconstriction in mesenteric arteries from PXR+/+ and PXR-/- mice.

To investigate the possibility that CYP epoxygenases were contributing to pregnancy induced alterations in vascular function, we used MS-PPOH to block all CYP epoxygenase activity. Treatment with this inhibitor did not alter the PE EC_{50} in any of the treatment groups. However it did cause a small but significant decrease in vasoconstriction at high does of PE in arteries from pregnant PXR+/+ mice. (EC_{80} PE alone: 10.5 ± 1.8 μM vs. PE+MSPPOH: 18.8 ± 1.8 μM, p=0.026; figure 14). To determine if one specific epoxygenase isoform, Cyp2c37, was contributing to the regulation of vascular tone, we used SUL to inhibit only Cyp2c37. There was no change in vasoconstriction at any concentration of PE in any of the treatment groups (figure 15).

14,15-EEZE is an EET antagonist that can block the effects of all EET regioisomers but is most specific for 14,15-EET. When we exposed vessels from non-pregnant and pregnant mice to 14,15-EEZE, there was no difference in the response to PE in vessels from non-pregnant PXR+/+, pregnant PXR+/+ or non-pregnant PXR-/- mice. In vessels from pregnant PXR-/- mice, however, treatment with 14,15-EEZE significantly inhibited vasoconstriction at low doses of PE (PE alone EC_{20}: 2.32 ± 0.28 μM vs. PE + 14,15-EEZE EC_{20}: 3.52 ±0.36 μM, p=0.028; figure 16).

4. PXR and *Cyp2c29* mRNA expression in mesenteric arteries from non-pregnant and pregnant PXR+/+ and PXR-/- mice.

We used qRT-PCR to determine if PXR was expressed in mesenteric arteries from non-pregnant and pregnant PXR+/+ and PXR-/- mice. All PXR+/+ samples were positive for PXR expression (table 3), indicating that PXR mRNA is present in mouse mesenteric arteries. No signal was detected in any of the samples from PXR-/- mice, confirming the PXR-/- genotype. To compare relative expression of PXR between groups, samples were normalized against 18S expression (table 4). There was no statistically significant difference between the level of PXR expression in mesenteric arteries from non-pregnant and pregnant mice (figure 17). However, there was considerable biological variability between the samples tested.

We also used qRT-PCR to evaluate *Cyp2c29* expression in the same tissue samples (figure 18). Unlike the PXR primers, the *Cyp2c29* primer set was not designed to cross an exon boundary, therefore there was the potential for this primer set to amplify genomic DNA. To ensure that any signal detected in the positive RT reactions was truly a reflection of gene expression, samples without reverse transcriptase were run as a control for each sample tested. A sample was considered positive if there was greater than 5 cycles difference between the positive RT and the no RT reactions. Three samples met this criteria, two from the non-pregnant PXR+/+ group and one from the preg PXR+/+ group. Based on these samples we can state that *Cyp2c29* is expressed in mouse mesenteric arteries. Since the signal in all of the other groups was quite low, there was there was not sufficient difference between the experimental samples and the no-RT controls to draw any conclusions about *Cyp2c29* expression in these samples (table 5).

Table 3 **Quantitative RT-PCR results: threshold cycles for PXR mRNA**

PCR reactions were performed in triplicate for each sample. C_T values are expressed as an average of the three reactions.

SAMPLE	AVERAGE C_T
Non-Preg PXR+/+	
1	29.9
2	31.9
3	32.0
Preg PXR+/+	
1	29.3
2	35.4
3	38.1

Table 4 **Quantitative RT-PCR results: threshold cycles for 18S mRNA**

PCR reactions were performed in triplicate for each sample. C_T values are expressed as an average of the three reactions.

SAMPLE	SAMPLE
Non-Preg PXR+/+	
1	9.9
2	9.1
3	9.1
Preg PXR+/+	
1	9.4
2	9.4
3	9.2
Non-Preg PXR-/-	
1	9.9
2	9.2
3	9.1
Preg PXR-/-	
1	8.9
2	9.5
3	9.4

Table 5 **Quantitative RT-PCR results: threshold cycles for *Cyp2c29* mRNA**

PCR reactions were performed in triplicate for each sample. C_T values are expressed as an average of the three reactions. * indicates > 5 cycles difference between the sample C_T and the C_T of the negative control.

SAMPLE	AVERAGE C_T OF SAMPLE TRIPLICATES	AVERAGE C_T OF NEGATIVE CONTROL TRIPLICATES
Non-Preg PXR+/+		
1*	25.6	34.5
2	28.6	32.4
3*	28.0	N/A
Preg PXR+/+		
1*	25.0	35.2
2	33.9	36.5
3	32.2	33.3
Non-Preg PXR-/-		
1	27.5	N/A
2	32.1	34.4
3	30.0	34.0
Preg PXR-/-		
1	31.8	35.7
2	32.7	33.4
3	34.6	36.7

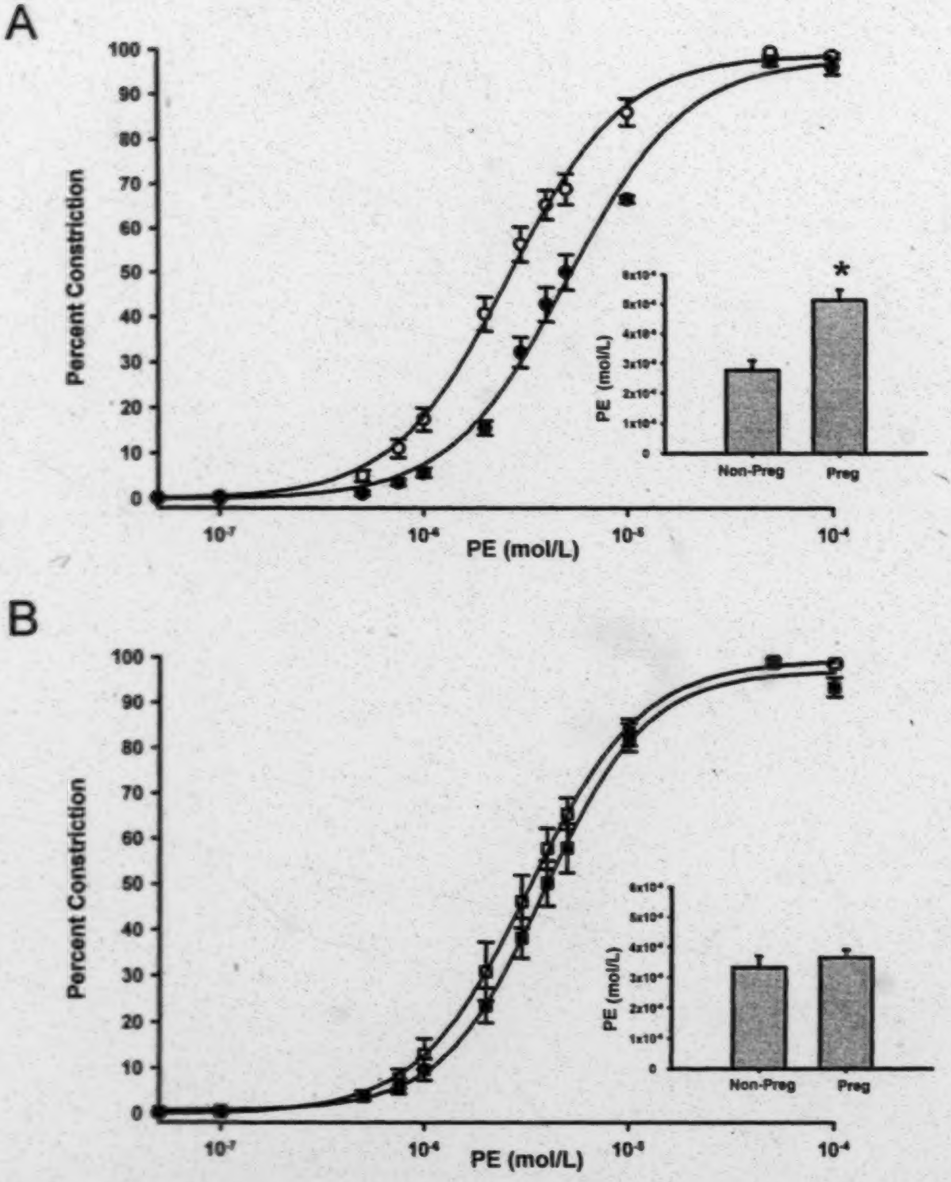


Figure 12 **Response of vessels from non-pregnant and pregnant mice to PE induced vasoconstriction.**

Adrenergic response in vessels from **A)** non-pregnant (○, n=8) and pregnant (●, n=8) PXR+/+ mice and **B)** non-pregnant (□, n=7) and pregnant (■, n=8) PXR-/- mice. *Insets*: PE EC_{50} responses of non-pregnant and pregnant mice calculated as mean ± SEM. *$p<0.05$ by t-test.

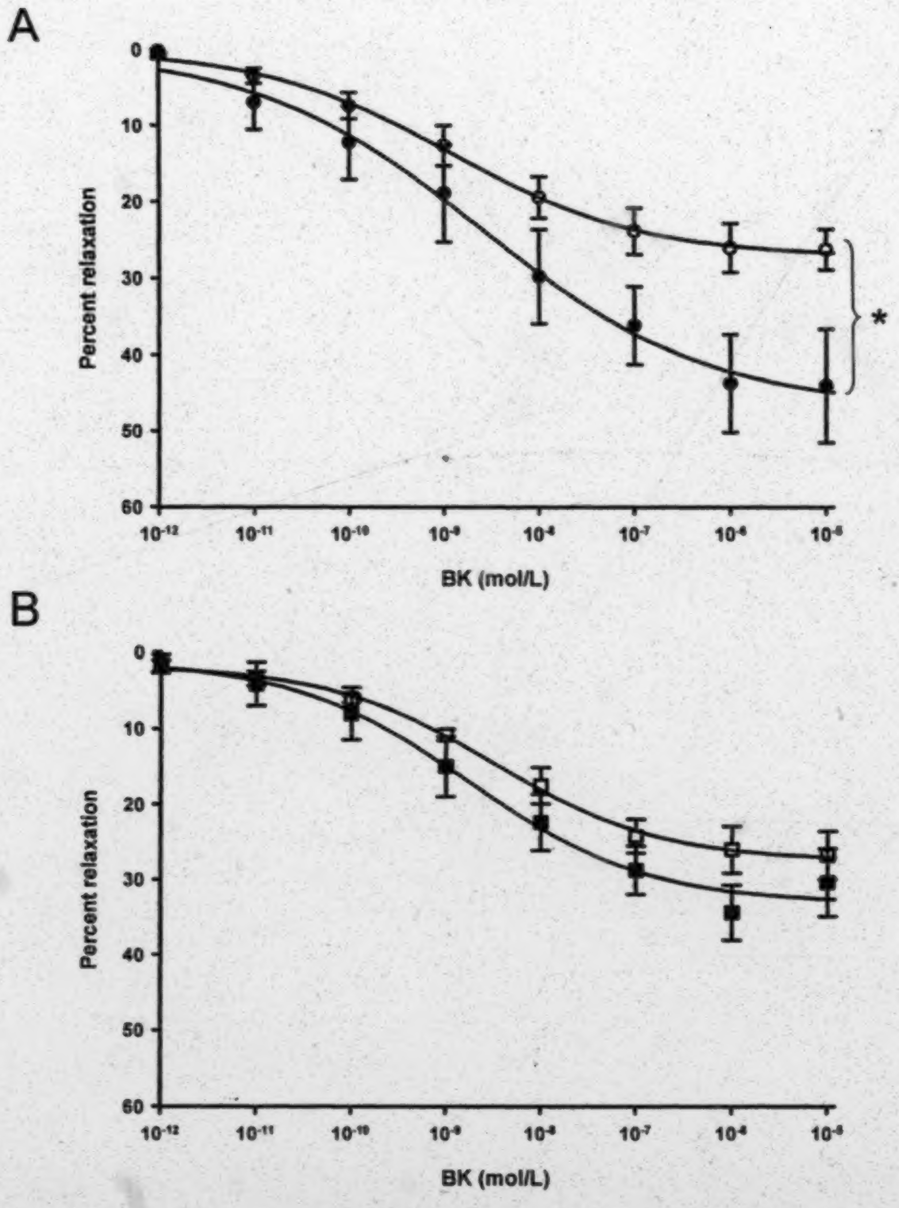


Figure 13 **Response of vessels from non-pregnant and pregnant mice to BK induced vasorelaxation.**

A) Endothelial dependent relaxation response of vessels from non-pregnant (○, n=6) and pregnant (●, n=6) PXR+/+ mice. B) Endothelial dependent relaxation response of vessels from non-pregnant (□, n=3) and pregnant (■, n=5) PXR-/- mice. Values are reported as mean ± SEM. *$p<0.05$ by 2 way repeated measures ANOVA.

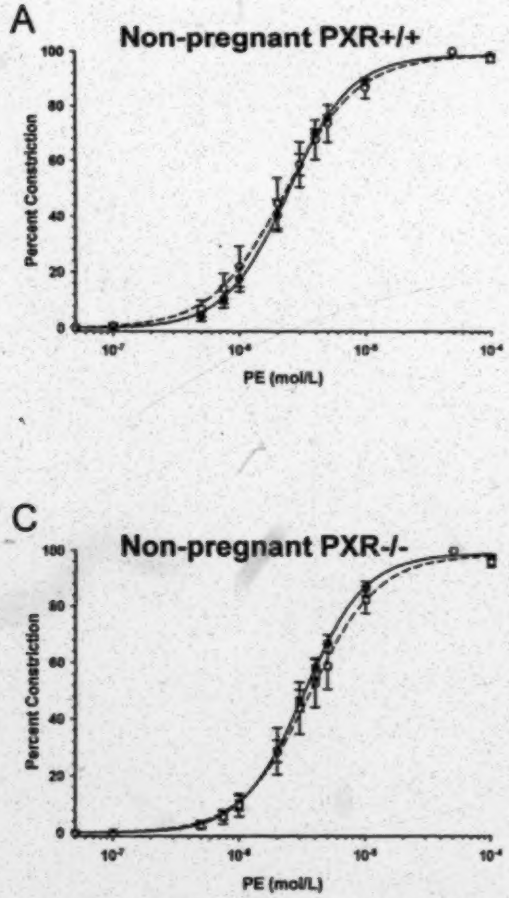


Figure 14 Effect of epoxygenase inhibition with MS-PPOH on PE induced vasoconstriction.

A) non-pregnant PXR+/+ mice treated with PE alone (●, n=6) or PE+MS-PPOH (○, n=4)
B) pregnant PXR+/+ mice treated with PE alone (●, n=6) or PE+MS-PPOH (○, n=5)
C) non-pregnant PXR-/- mice treated with PE alone (■, n=7) or PE+MS-PPOH (□, n=5)
D) pregnant PXR-/- mice treated with PE alone (■, n=6) or PE+MS-PPOH (□, n=5)
MS-PPOH used at 50 μM. Values represent mean ± SEM. $^*EC_{80}$ $p<0.05$ by t-test.

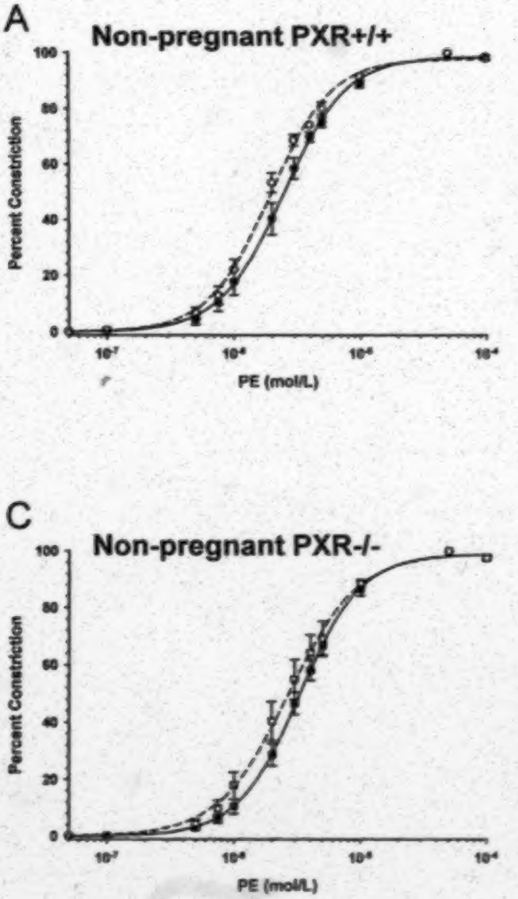


Figure 15 **Effect of Cyp2c37 inhibition with SUL on PE induced vasoconstriction.**

A) non-pregnant PXR+/+ mice treated with PE alone (●, n=7) or PE+SUL (○, n=3)
B) pregnant PXR+/+ mice treated with PE alone (●, n=6) or PE+SUL (○, n=4)
C) non-pregnant PXR-/- mice treated with PE alone (■, n=7) or PE+SUL (□, n=8)
D) pregnant PXR -/- mice treated with PE alone (■, n=6) or PE+SUL(□, n=5)
SUL used at 20μM. Values represent mean ± SEM.

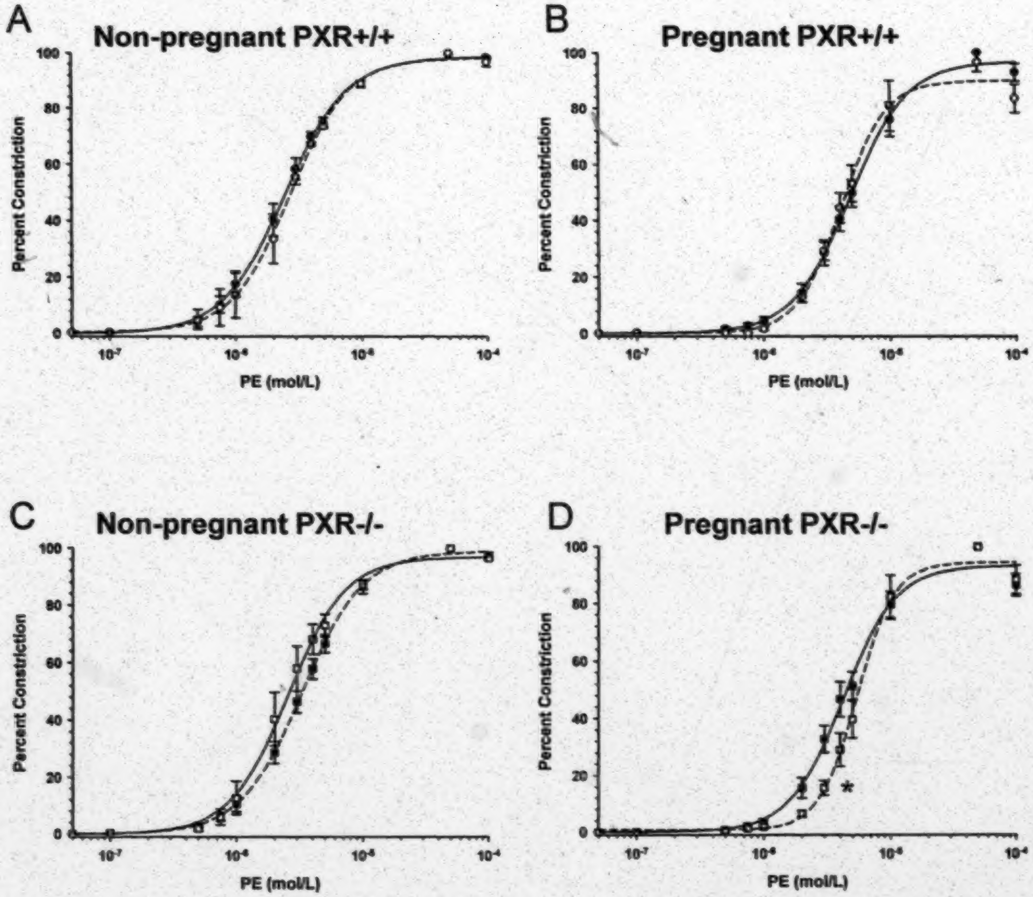


Figure 16 Effect of EET inhibition with 14,15-EEZE on PE induced vasoconstriction.

A) non-pregnant PXR+/+ mice treated with PE alone (•, n=7) or PE+14,15-EEZE (○, n=4) **B)** pregnant PXR+/+ mice treated with PE alone (•, n=6) or PE+14,15-EEZE (○, n=4) **C)** non-pregnant PXR-/- mice treated with PE alone (■, n=7) or PE+14,15-EEZE (□, n=7) **D)** pregnant PXR -/- mice treated with PE alone (■, n=6) or PE+14,15-EEZE (□, n=4). 14,15-EEZE used at 10μM. Values represent mean ± SEM. *EC_{20} $p<0.05$ by t-test.

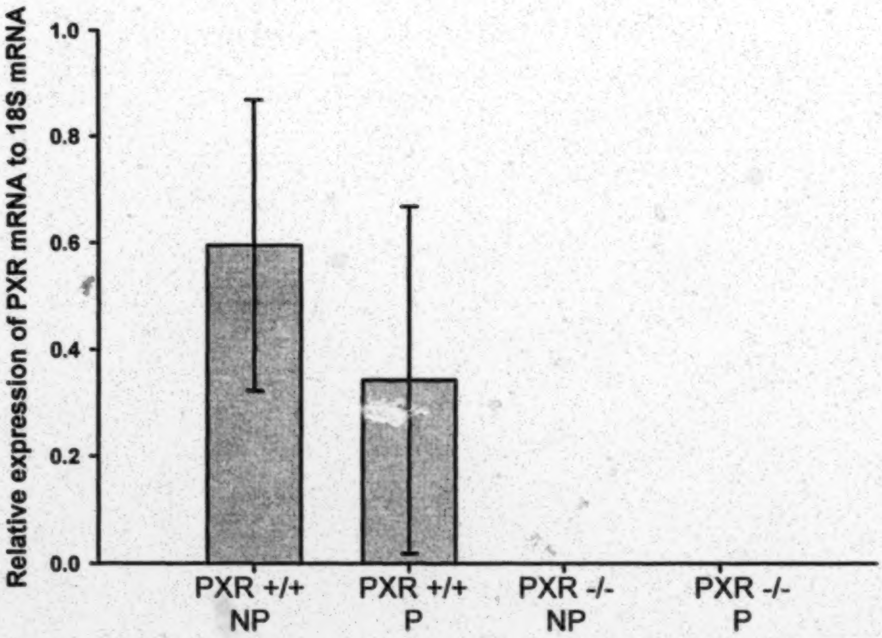


Figure 17 **Relative expression of PXR mRNA in mesenteric arteries from non-pregnant (NP) and pregnant (P) PXR+/+ and PXR-/-mice.**

RNA was extracted from three pools of mesenteric arteries (representing two mice each) and tested for PXR mRNA expression by qRT-PCR. Results are calculated as a relative expression ratio between PXR and 18S mRNA and reported as mean ± SEM.

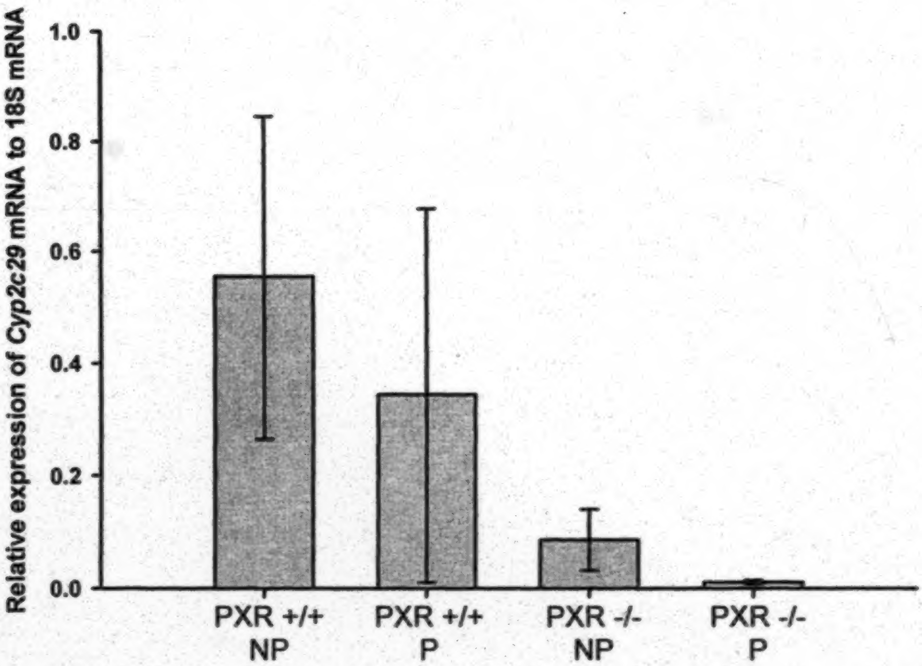


Figure 18 **Relative expression of *Cyp2c29* mRNA in mesenteric arteries from non-pregnant (NP) and pregnant (P) PXR+/+ and PXR-/- mice.**

RNA was extracted from three pools of mesenteric arteries (representing two mice each) and tested for *Cyp2c29* mRNA expression by qRT-PCR. Results are calculated as a relative expression ratio of *Cyp2C29* and 18S mRNA and reported as mean ± SEM.

CHAPTER IV - DISCUSSION

1. Role of PXR in the mediation of vascular function

Alterations in maternal vascular function are necessary during gestation yet the mechanisms underlying these pregnancy induced adaptations remain poorly understood. We hypothesized that PXR, activated by progesterone metabolites, provided the means by which changes in vascular function were initiated in response to pregnancy. Small mesenteric arteries were chosen for analysis because they are known to contribute to the regulation of blood pressure[171] and because the contribution of EDHF to vascular tone is proportionally greater in resistance sized arteries relative than in larger vessels[16]. Our results show a decreased sensitivity to PE and an enhanced response to BK in vessels from pregnant PXR+/+ mice. In PXR-/- mice, however, presumably due to the absence of a functional PXR protein, these pregnancy-induced alterations did not occur, indicating that PXR contributes to the regulation of vascular tone during pregnancy.

In support of this conclusion, other members of the Davidge laboratory found that treatment with the progesterone metabolite, 5β-DHP, induced the same vascular responses in PXR+/+ and PXR-/- mice that occur in pregnancy. 5β-DHP was chosen as the tool to activate PXR because of its potency as a PXR ligand and because plasma levels of this steroid are known to rise during pregnancy[172]. This methodology also had the advantage of allowing the effect of PXR activation on vascular function to be isolated and examined independently of any of the other potentially confounding physiological changes which occur during gestation. In these experiments, slow release pellets containing either 5β-DHP or a placebo were implanted subcutaneously into PXR+/+ and PXR-/- mice. Seven days later, animals were then euthanized, vessels were harvested and their vasorelaxation response to methylcholine was assessed using wire myography. After one week of exposure, vessels from PXR+/+ mice treated with 5β-DHP showed a significant increase in methacholine induced vasorelaxation relative to those animals treated with placebo pellets. In contrast, there was no difference in the vasorelaxation response of vessels from PXR-/- mice treated with 5β-DHP as compared to those who received the placebo (data not shown). The observation that exposure to an exogenous progesterone metabolite stimulated enhanced vasorelaxation in PXR+/+ but not PXR-/- mice further confirms a role for PXR in the alteration of vascular tone and suggests that the vascular changes which occur during pregnancy may be the result of increases in progesterone metabolites.

These findings reveal a novel role for PXR, and may represent an important new perspective in our understanding of how the maternal vasculature adapts to the demands of pregnancy. Moreover, it may also have broader implication relative to our understanding of vascular physiology in general. As was indicated previously, gender differences exist in the relative contribution of EDHF to the regulation of vascular tone[58-60]. The authors of these studies discuss the possibility that these differences are mediated by sex hormones, likely estrogen. In light of our data, an additional explanation may be that these differences are driven by the progesterone and progesterone metabolites which are present in cycling females.

2. Mechanism of PXR mediated, pregnancy-induced changes in vascular function

Having shown that PXR mediates vascular function, we next investigated the signaling pathways through which this might be occurring. Since PXR can regulate the expression of CYP epoxygenases, we questioned whether PXR dependent changes in vascular function were mediated by CYPs. In order to gain as much knowledge as possible about CYP epoxygenases and EETs, we chose three pharmacological agents, each of which acts on a different aspect of the putative PXR-CYP-EET signaling pathway, to pharmacologically isolate the influence of CYP epoxygenases and EETs. MS-PPOH, because it inhibits all CYP epoxygenases allowed us to determine the effects of EET production on vascular function. SUL, the specific CYP2C9 inhibitor was chosen because this isoform, and presumably its murine orthologue, Cyp2c37, has been reported to be involved in mediating the EDHF response. Finally, 14,15-EEZE was chosen because it can antagonize all EETs, although it is most effective against 14,15-EET.

Ideally, we would have assessed the contribution of CYP epoxygenases and EETs to both the vasoconstriction and vasorelaxation responses. Unfortunately however, the great tendency for vessels from pregnant mice to spontaneously relax made this unfeasible since the inhibitors had to be applied before PE was added, yet we could not know prior to preconstriction which vessels would have a suitable preconstriction plateau. Therefore, we were restricted to investigating the role of CYPs/EETs in the context of vasoconstriction only. This inability to examine the contribution of CYPs and EETs to vasorelaxation was due to characteristics inherent to the function of mouse mesenteric arteries during pregnancy and was unavoidable, although it did represent a limitation of these studies. Nevertheless, investigating the involvement of this signaling pathway in the regulation of vasoconstriction still provide valuable knowledge and insight regarding vascular function in the PXR-/- mouse model and in pregnancy in general.

2.1. Involvement of CYP epoxygenases in PXR mediated, pregnancy-induced changes in vascular function

Relative to the effects of CYP inhibition on vascular function, we hypothesized that treatment with MS-PPOH would increase vasoconstriction. We found that treatment MS-PPOH had no effect on the PE EC_{50} of either non-pregnant or pregnant PXR+/+ or PXR-/- mice, implying that CYP epoxygenases do not contribute to the differential regulation of vasoconstriction during pregnancy in this model. MS-PPOH inhibition, however, did cause a small but significant *decrease* in vasoconstriction at high does of PE in arteries from pregnant PXR+/+ mice but not in any of the other groups. Recently, it has been reported that *in vivo* CYP inhibition by fluconazole alone did not alter basal radial artery diameter in human male subjects[173] but administration of fluconazole combined with a NOS inhibitor significantly decreased radial artery diameter. The observation that the effect of CYP inhibition can be masked by NO may help explain the lack of effect of MS-PPOH on vasoconstriction in our study, since we did not use MS-PPOH in conjunction with a NOS inhibitor. Interestingly, the authors also report that fluconazole alone induced a slight increase in radial artery blood flow which they attribute to the suppression of basal release of reactive oxygen species which are produced as a byproduct of CYP activity[119]. Since we did not directly test for the presence of reactive oxygen species, we can not say for certain that this is the reason for the decrease in vasoconstriction seen at high does of PE in arteries from pregnant PXR+/+ mice, however this seems a likely explanation. Most importantly, however, regardless of the fact that MS-PPOH diminished vasoconstriction, the fact that this response occurred only in the arteries of pregnant PXR +/+ mice but not in any of the other groups lends further support to the conclusion that PXR is activated during pregnancy.

2.2. Involvement of *Cyp2c37* in PXR mediated, pregnancy-induced changes in vascular function

Because SUL has been used extensively by other groups who have shown that CYPs, specifically CYP2C8/9 (mouse Cyp2c29/37) are involved in the EDHF response, we also used SUL in our experiments to investigate the involvement of this CYP isoform in PXR mediated, pregnancy induced changes in vascular function. We found that there was absolutely no effect of SUL on the response to α_1-adrenergic stimulation in any of the groups at any concentration of PE. This suggests that Cyp2c29/37 does not contribute to mediation of vasoconstriction in these animals. Alternately, it is possible that, in the mouse, SUL does not inhibit these CYP isoforms. Very few studies have used SUL to inhibit the mouse equivalents of CYP2C8/9. In accord with our results, Ding et al. also found that SUL did not alter BK induce relaxation in mouse hearts, although other CYP inhibitors (clotrimazole, miconazole, 17-ODYA) did[174]. Further investigation is required to resolve this discrepancy, although, given that inhibition with MS-PPOH had only minor effects on the vasoconstriction response, it is likely that the Cyp2c29/37 isoforms are not involved in the regulation of vasoconstriction in this model.

2.3. Involvement of EETs in PXR mediated, pregnancy-induced changes in vascular function

14,15-EEZE is a structural analogue of 14,15-EET[175] which can antagonize all EET regioisomers but is most effective at blocking the activity of 14,15-EET. When we exposed vessels to this inhibitor, we found that it decreased PE-induced vasoconstriction in vessels from pregnant PXR-/- animals but not from any of the other groups, albeit at only low concentrations (EC_{20}) of PE. This finding is again contrary to our hypothesis, which proposed that EETs mediate vasodilation during pregnancy and would have led us to expect an increase in vasoconstriction in the pregnant PXR+/+ group. The observation that vasoconstriction was decreased instead of increased would suggest that, in this situation, EETs are acting as vasoconstrictors. Although EETs are largely considered to be vasodilators, there is evidence that they can also act as vasoconstrictors. For example, all EETs but especially 5,6-EET, cause vasoconstriction in small diameter intralobular pulmonary arteries from rabbits even though in non-resistance sized extralobular pulmonary arteries they cause vasodilation[176]. Furthermore, Fang et al. have also shown that while 14,15-EET could produce relaxation in preconstricted porcine coronary artery rings, it could also cause vasoconstriction[177]. Alternately, the observation that 14,15-EEZE decreased constriction in mouse mesenteric arteries could be attributed to the fact that 14,15-EEZE has been shown to have some vasodilatory properties[188].

The observation that 14,15-EEZE reduced PE-induced vasoconstriction only in vessels from pregnant PXR-/- mice was also puzzling, but can possibly be explained in light of the fact that PXR-/- mice do not develop enhanced relaxation in response to pregnancy. Thus, it is possible that EETs were also acting as constrictors in vessels from PXR+/+ pregnant mice and that this activity was being blocked by 14,15-EET but that the effects of this inhibition were so small relative to the massive increases in vasorelaxing molecules during pregnancy that any changes were masked. Again, this observation does not rule out the possibility that EETs may act as vasodilators upon direct endothelial stimulation with a vasorelaxing agent since they may interact with different downstream molecules, in a manner similar to that by which epinephrine can cause both vasoconstriction and vasodilation by acting on different receptor subtypes.

In summary, the effect of inhibition with MS-PPOH, SUL and 14,15-EEZE on PE-induced vasoconstriction was minimal overall. This highlights difference between the regulation of PE-induced vasoconstriction, which acts via stimulation of α-adrenergic receptors on the VSM, and BK-induced vasorelaxation, which requires the direct stimulation and activation of the endothelium to alter VSM tone. We had hypothesized that the increased activation of PXR during pregnancy would blunt vasoconstriction by increasing the amount of CYP produced vasodilators present in the paracrine milieu even in the basal state. However, this does not appear to be the case. It is important to note, however, the lack of CYP/EET involvement in the regulation of vasoconstriction does not exclude the possibility that this pathway may be involved in the mediation of vasorelaxation, since there is some suggestion that CYP activity requires the release of arachidonic acid by phospholipase A_2 and/or an increase in endothelial Ca^{++} [178]. Thus, we can conclude that PXR-dependent mediation of vasoconstriction and vasodilation occur via different mechanisms, and that CYPs/EETs do not appear to be involved in the mediation of vasoconstriction during pregnancy. An alternate possibility is that PXR may be acting via the induction of other vasoregulatory molecules. In particular, PXR has been shown to upregulate the expression of iNOS[10]. Information in this area is limited, however, and further investigation is required to determine if PXR-mediated regulation of vascular function may also be acting through the induction of NO.

3. PXR and *Cyp* expression in mouse mesenteric arteries

3.1. Changes in PXR expression in the vasculature during pregnancy

Little is known about the effect of pregnancy on maternal PXR expression. We found no difference between the levels of expression of PXR mRNA in mesenteric arteries from non-pregnant compared to pregnant mice, although there was a large degree of variability of in our samples. Previously, Masuyama et al reported that in the liver and ovaries of pregnant BALB/cA mice the expression of PXR mRNA was increased fifty-fold over non-pregnant controls[131]. Recently, however, Sweeny et al. have shown that, in C57BL/6 mice, expression of PXR in hepatic tissue decreases near term[179]. Reasons for these differences in expression are as yet undetermined, although they could reflect differences in the strain of mouse used and or the period of gestation during which the samples were collected. Nevertheless, regardless of the direction of change in the level of PXR expression, the presence of PXR mRNA in the vascular tissue in our model lends further support to our hypothesis that PXR mediates vascular tone.

3.2. Changes in *Cyp2c29* expression in the vasculature during pregnancy

Since we hypothesized that PXR was acting through CYP epoxygenases to mediate changes in vascular tone in response to pregnancy, we wanted to assess CYP expression in our model. Ideally, we would have liked to analyze the mouse mesenteric arteries for expression of multiple CYP epoxygenases at once. Since we did not know which CYP epoxygenase, if any, was induced by PXR in these experiments, it would have been desirable to assess the expression of all epoxygenases which are known to be present in the vasculature. However, owing to the small amount of tissue available, we could only assess 2 genes of interest in addition to the housekeeping gene. We therefore chose *Cyp2c29* since that primer was commercially available and since that isoform is orthologous to CYP2C8 which had been implicated in the EDHF response. Regardless, even though the information gained was not as complete as it could have been, these investigations still provided us with important insight upon which to base future studies.

Only three of the 12 samples tested were positive for *Cyp2c29* expression. Thus, while we can conclude that CYP is expressed in mouse mesenteric arteries, the expression is low. Although the large degree of variability in our samples made it difficult to draw any conclusions regarding changes in CYP expression, we can say that it appears that CYP expression is higher in PXR+/+ than in PXR-/- animals and that, if anything, the trend is for CYP expression to decrease in PXR+/+ mice in response to pregnancy. Pregnancy is known to cause a decrease in overall hepatic CYP expression[180]. Recently this pattern has also been confirmed for a number of individual rat CYP isoforms in the liver including CYP2C6 (equivalent to human

CYP2C9) which was shown to be decreased from mid-pregnancy onwards compared to non-pregnant controls[181]. The reasons underlying this downregulation of CYP expression during pregnancy remain unclear, however, it has been suggested that this is possibly a result of oxidative stress, due to elevated NO production, or a direct inhibition of CYPs by progesterone in order to sustain the high plasma progesterone concentrations necessary to maintain pregnancy[180, 181]. In contrast, CYP expression and epoxygenase activity has been shown to increase in rat renal microsomes as gestation progresses [127]. The reason for these differences is unclear, although likely is a reflection of tissue specificity of CYP expression.

4. Summary

In summary, we have shown that PXR mediates vascular changes during pregnancy by promoting blunted vasoconstriction and enhanced vasorelaxation. However, these effects of PXR on vasoconstriction do not appear to due to the activity of CYPs or EETs. PXR is expressed in vascular tissue in the mouse as is *Cyp2c29*, albeit at very low levels. Biological variability amongst the samples tested prevented the comparison of gene expression between the non-pregnant and pregnant states. It is important to remember, however, that the mechanisms by which vasoconstriction and vasorelaxation are regulated differ, that levels of mRNA expression may not necessarily be indicative of protein level or activity, and that gene expression late in gestation may not necessarily be reflective of expression in early or mid gestation. Thus, these observations do not exclude the possibility that PXR may promote vasorelaxation via CYP dependent production of vasodilatory EETs, although other possibilities, such as the fact that PXR can also induce iNOS should be examined.

5. Future Directions

Based on the results generated by this work, a number of other investigations are proposed including:

- Examination of blood pressure changes during pregnancy in PXR+/+ and PXR-/- mice. Interestingly, a lack of PXR does not alter the fertility or gestation of the PXR-/- mouse. It is possible that while PXR appears to be necessary for vascular adaptations to pregnancy to occur, redundant mechanisms may exist within the mouse which allow for compensation in these animals. Nevertheless, the fact that the usual vascular adaptations to pregnancy fail to develop in the PXR-/- mouse would suggest that these animals would be likely to be hypertensive. Therefore, blood pressure measurements taken throughout pregnancy in both PXR+/+ and PXR-/- animals are recommended to gain further insight into the PXR-/- phenotype.

- Assessment of vascular function should be conducted in a more robust model, such as the rat. Although the power of the PXR knockout model is lost if studies are conducted in the rat, it is important to assess the mechanism of PXR-mediated regulation of vasorelaxation during pregnancy. Pharmacological inhibition/induction of PXR in the rat could provide a complementary model for the mouse studies and allow for a broader investigation of PXR-mediated regulation of vascular function. Inhibitory agents could also be used in this model to investigate other aspects of PXR-mediated vascular regulation, including the effects of NO and PGI_2 inhibition on PXR-mediated vasorelaxation responses, and whether or not PXR is acting through iNOS. Finally, the contribution of CYP produced reactive oxygen species to the regulation of vascular tone could also be assessed with the aim of determining if the decreased vasoconstriction seen upon treatment with MS-PPOH occurred due to alterations in CYP production of reactive oxygen species.

- Continued examination of gene expression in mouse mesenteric arteries during pregnancy. Increasing the size of the experimental groups will allow changes in expression of PXR and *Cyp2c29* in response to pregnancy to be precisely determined. Additionally, the expression of other *Cyp* isoforms, such as members of the CYP2J family, and iNOS could also be investigated.

6. Conclusions and Perspectives

This work has established a novel role for the nuclear receptor, PXR, in the regulation of vascular tone, and has provide a basis for further investigations into the mechanisms by which PXR is acting to alter vasorelaxation and vasoconstriction. Overall, this new role establishes PXR as a link between the state of pregnancy and the vascular changes which occur during gestation, and as such may contribute to an improved understanding of how maternal vascular adaptations to pregnancy occur. Most importantly, application of these findings may provide insight and new avenues of investigation for clinical conditions including vascular dysfunction during pregnancy as well as the cardioprotective effects of gender differences on vascular function.

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